The Relationship Between the Success of Invasive Plants and their Mode of Reproduction

Dissertation

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Publications

Sailer C, Schmid B, Stöcklin J, Grossniklaus U, Sexual *Hieracium pilosella* plants are better between-species, while apomictic plants are better within-species competitor. *submitted*

Sailer C, Stöcklin J, Grossniklaus U, Behaviour of apomictic and sexual siblings in different competition settings, *in preparation*

Sailer C, Stöcklin J, Grossniklaus U, Dynamics of apomixis and sexuality on an alpine glacial foreland, *in preparation*

Heike Lindner, Michael T. Raissig, Christian Sailer, Hiroko Shimosato-Asano, Rémy Bruggmann, and Ueli Grossniklaus (2012) SNP-Ratio Mapping (SRM): Identifying Lethal Alleles and Mutations in Complex Genetic Backgrounds by Next-Generation Sequencing. Genetics, Vol. 191, 1381–1386. doi: 10.1534/genetics.112.141341

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Singh A, Bashir T, Sailer C, Maharasi A, Dhanapal S, Grossniklaus U, Baskar R, Parental age effects on somatic mutation rates in flowering plants, *submitted*

Talks

Sexuals compete better than apomicts, but apomicts are bigger, 2013 LS^2 annual meeting, (R)Evolutions in biology, Zürich

Asexuelle Vermehrung in Planzen: Was ist Apomixis?, Vorträge Wintersemester 2012/13, Zürcher Botanische Gesellschaft

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Thesis Abstract

Apomixis is the asexual reproduction through seed. It can be seen as a deregulation of 3 sexual processes in space and time. These processes are meiosis, which is avoided and hence referred to as apomeiosis, and fertilization, which does not occur and the embryo develops parthenogenetically. The third process refers to endosperm development which can either require triggering by fertilization, as in sexual reproduction, or it develops autonomously. Several different types of apomixis are distinguished and most result in fixation of the maternal genetic constitution. Thinking of the benefit of apomixis for applied research, fixation of the genetic constitution of the mother is thought to fix associated, complex phenotypes as, for example, heterosis. From an ecological perspective, two apparently opposite effects arise. On the one hand, apomeiosis disables removal of mutations from a population, which in turn leads to accumulation of mutations, which results in extinction of genotypes once a critical number of deleterious mutations has been reached (Muller's ratchet). On the other hand, independence of fertilization provides reproductive assurance, and consequently an advantage in colonizing sparsely populated habitats (Baker's law, Tomlinson's prediction), as is the case in succession, or sometimes during invasion of new habitats.

To address the question of the role of apomixis in invasion, apomicts and sexuals were phenotypically compared under different competition settings. To test if apomixis is advantageous in sparse population densities, seeds were collected from one apomictic species (*Hieracium pilosella* L.) along a successional gradient and analyzed for its developmental origin. To answer the question of fixation of phenotypes across apomictic generations, new apomictic lines were generated and different generations were grown at the same time under equal conditions and phenotyped.

Phenotypic differences between apomicts and sexuals under different competition settings were not only due to different modes of reproduction, but were always dependent on the rest of the genome, the genetic background. More apomictic offspring occurred at early stages of succession. Furthermore, the level of apomixis was low in general, but it was highly variable. In addition, two triploid patches, which were the result of n + 0 offspring, were found at early stages of succession, despite a bias against n + 0 offspring. Additionally, apomictic fertility was highly variable between different apomictic genotypes, and apomicts had a smaller floral display than sexuals.

The results from competition experiments suggest that apomixis does not confer a fitness advantage *per se*. However, apomixis is advantageous in sparse population

densities, which supports Tomlinson's prediction. In addition, the occurrence of triploid patches, which are the result of a rare event, supports Baker's law. The results from a natural population and from different apomictic lines generated in this work show that apomixis is facultative and a quantitative trait. Work is still underway to address the question of wether apomixis would lead to fixation of phenotypes over several generations. At equilibrium with sexual reproduction, apomixis does add to the diversity of populations and its main advantage is reproductive assurance.

Zusammenfassung der Dissertation

Apomixis ist die asexuelle Vermehrung über Samen. Sie kann als Deregulation von drei sexuellen Prozessen in Raum und Zeit angesehen werden. Diese Prozesse sind Meiose, welche vermieden und was als Apomeiose bezeichnet wird, sowie Fertilisation, welche nicht erfolgt und der Embryo entwickelt sich daher parthenogenetisch. Der dritte Prozess betrifft die Entwicklung des Endosperms. Diese kann entweder durch Fertilisation ausgelöst werden, wie bei sexueller Vermehrung, oder das Endosperm entwickelt sich autonom. Mehrere verschiedene Arten von Apomixis werden unterschieden und die meisten fixieren die mütterliche genetische Konstitution. Daraus ergibt sich auch eine Anwendung für Apomixis, denn eine fixierte mütterliche genetische Konstitution könnte komplexe Phenotypen, wie zum Beispiel Heterosis, fixieren. Ökologisch betrachtet ergeben sich dadurch zwei gegensätzliche Effekte. Einerseits führt Apomeiose zur Anhäufung von Mutationen, welche zum Aussterben eines Genotyps führen können, sobald eine kritische Anzahl an schädlichen Mutationen erreicht ist (Mullers Ratsche). Andererseits führt die Unabhängigkeit von der Fertilisation zu reproduktiver Absicherung und als Folge zu Vorteilen bei der Kolonialisierung von dünn besiedelten Habitaten (Bakers Gesetz, Tomlinsons Voraussage), wie sie zum Beispiel in Sukzession oder manchmal während der Invasion von neuen Habitaten auftreten.

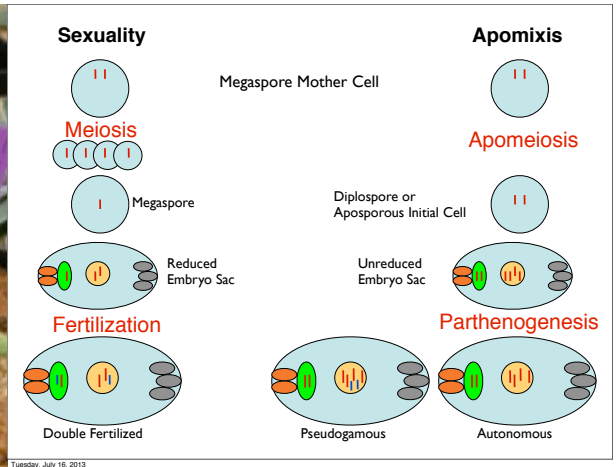
Um die Rolle von Apomixis während der Invasion zu untersuchen, wurden Apomikten und Sexuelle phenotypisch verglichen, und zwar unter verschiedenen Konkurrenzbedingungen. Um zu sehen, ob Apomikten tatsächlich einen Vorteil in dünn besiedelten Habitaten haben, wurden Samen von einer apomiktischen Art entlang eines primären Sukzessionsgradienten gesammelt und auf ihren Entwicklungsursprung untersucht. Um die Frage zu beantworten, ob Phenotypen durch Apomixis über mehrere apomiktische Generationen fixiert werden können, wurden neue apomiktische Linien erzeugt, und unterschiedliche Generationen wurden gleichzeitig unter den selben Bedingungen gezogen und phenotypisiert.

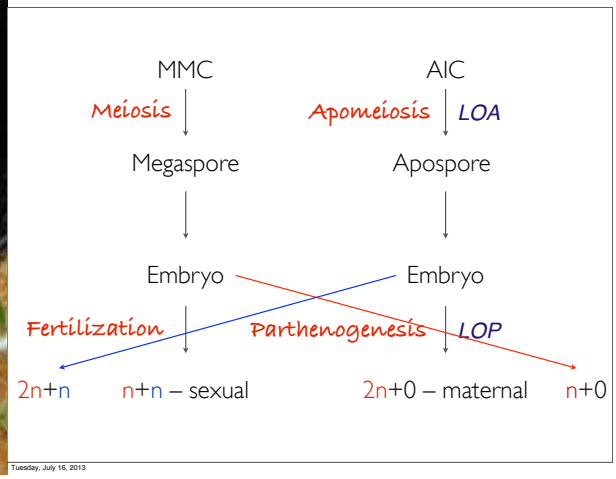
Phenotypische Unterschiede zwischen Apomikten und Sexuellen hingen nicht nur von der Art der Reproduktion, sondern auch vom restlichen Genom, dem unabhängigen Genotyp, ab. Mehr apomiktische Nachkommen wurden in frühen Sukzessionsstadien gefunden. Ausserdem war das Niveau von Apomixis im Allgemeinen gering, allerdings zeigte es grosse Variation. Zusätzlich wurden zwei triploide Felder, welche durch n + 0 Nachkommen gebildet wurden, in frühen Sukzessionsstadien gefunden, obwohl es eine Tendenz gegen n + 0 Nachkommen gab. Zusätzlich variierte die apomiktische Fertilität

stark zwischen verschiedenen Genotypen, und Apomikten hatten kleinere Blütenköpfchen als Sexuelle.

Die Resultate der Konkurrenzexperimenten lassen darauf schliessen, dass Apomixis keinen Fitnessvorteil *per se* bringt. Trotzdem ist Apomixis vorteilhaft in dünn besiedelten Habitaten, was Tomlinsons Voraussage unterstützt. Zusätzlich unterstützt das Auftreten der triploiden Felder Bakers Gesetz. Die Ergebnisse der natürlichen Population und der unterschiedlichen apomiktischen Linien, welche im Verlauf dieser Arbeit erzeugt wurden, zeigen, dass Apomixis fakultativ und eine quantitative Eigenschaft ist. Derzeit wird noch an der Frage gearbeitet, ob Apomixis zur Fixierung von Phenotypen über mehrere Generationen führt. Ist Apomixis im Gleichgewicht mit sexueller Vermehrung, trägt Apomixis zur Vielfalt einer Population bei. Des Weiteren ist der Hauptvorteil von Apomixis in der reproduktiven Absicherung zu sehen.

General Introduction





There are more possibilities to reproduce than “common” sexual reproduction. Lower animals, for example hydra, can reproduce via budding. Some higher animals can also reproduce parthenogenetically, i.e. without fertilization. Budding corresponds to vegetative reproduction in plants. Furthermore, plants can reproduce asexually via seeds, i.e. without meiosis and fertilization. Plants are unique in terms of a single individual being capable to reproduce via all three different pathways.

First, distinct modes of reproduction and their differences are introduced, followed by evolutionary and ecological theories about the maintenance of the different modes of reproduction. Last, the used model organism, *Hieracium pilosella* L., will be introduced.

Sexuality

Sexual reproduction involves the regular alternation of gamete formation by meiosis and gamete fusion (fertilization) to form a zygote (Lincoln et al. 1998). In this thesis, I refer to sexual reproduction as sexuality. Sexuality goes hand in hand with the biological life cycle.

Life Cycle

The life cycle 1) is the sequence of events from the origin as a zygote, to the death of an individual, 2) are those stages through which an organism passes between the production of gametes by one generation and the production of gametes by the next (Lincoln et al. 1998).

Diploid plants alternate between diplophasic (2n, sporophyte) and haplophasic (n, gametophyte) states (following notation in Greilhuber et al. 2005) (Figure 1).

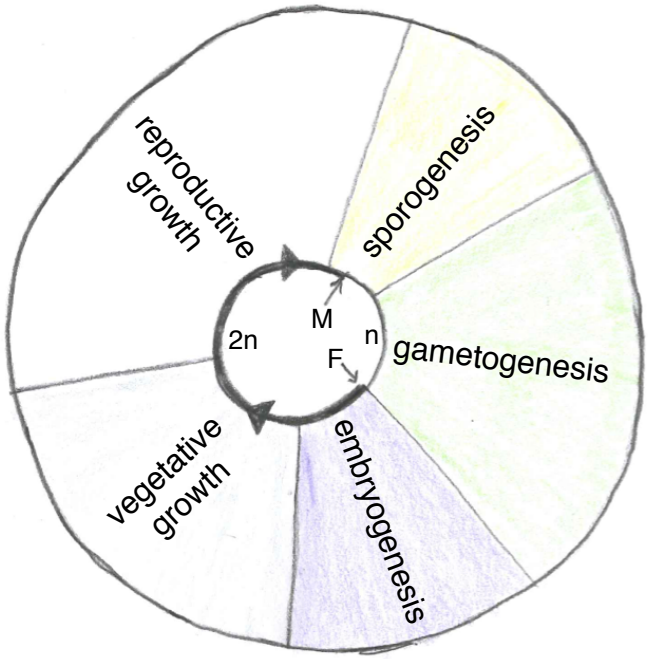


Figure 1. Life cycle
Sexually reproducing plants alternate between diplophasic and haplophasic states. Meiosis, which happens during sporogenesis (yellow) in plants, constitutes the transition from diplophase to haplophase. The meiotic products undergo gametogenesis (green), developing into mature gametophytes, which produce the gametes. Fertilization, which initiates embryogenesis (purple), causes the transition from haplophase to diplophase. The plant grows vegetatively (grey) until it reaches a certain maturity to transit into reproductive growth (white).
M – Meiosis; F – Fertilization; thick line, 2n – diplophase; thin line, n – haplophase; yellow – sporogenesis; green – gametogenesis; purple – embryogenesis. The two arrows indicate meiosis and fertilization, respectively.

In plants, the transition from diplophase to haplophase is called sporogenesis (Campbell and Reece 2002), which is followed by gametogenesis, during which the gametophytes develop (Campbell and Reece 2002, Wolpert et al. 2011). The transition from haplophase to diplophase happens at fertilization. Embryogenesis is the process of embryo development, which starts with fertilization, in which gametes fuse to form the zygote that further develops to form the embryo (Junshi 1994, Campbell 2002, Wolpert et al. 2011). In order to better understand these transitions and the development of organisms, mitotic cell division and meiosis have to be introduced first.

Mitosis

The mitotic cell cycle has four phases: 1) G1-phase, 2) S-phase (DNA replication), 3) G2-phase, 4) M-phase (chromatid segregation, normally followed by cell division). The M-phase (mitosis) is further subdivided into: i) prophase (chromosomes condense), ii) metaphase (condensed chromosomes align on the metaphase plate and are attached to the spindle), iii) anaphase (sister chromatids are segregated), iv) telophase (chromosomes are at opposing cell poles), and v) cytokinesis (cell division) (Figure 2).

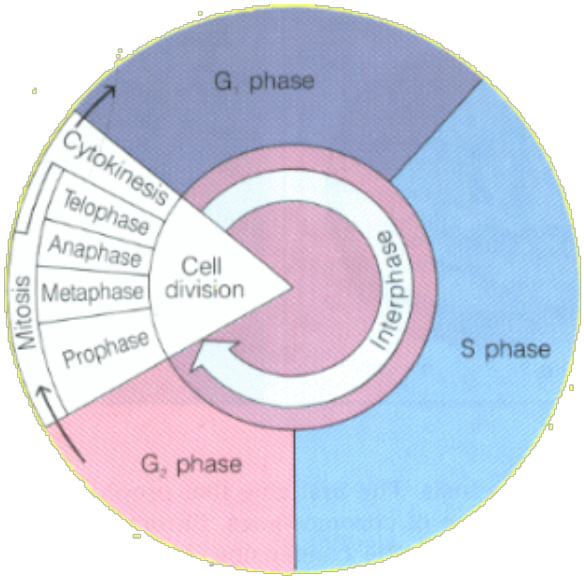


Figure 2. Cell cycle – Mitosis
G1-phase is the gap between cell division and DNA synthesis. During S-phase (Synthesis-phase), the genome is duplicated. G2-phase is the gap between S-phase and M-phase. G1-, S- and G2-phase together are called interphase. During M-phase (Mitosis-phase), chromosomes are segregated and the cell divides into two daughter cells. M-phase consists of 5 sub-phases. Figure taken from http://home.comcast.net/~clupold96/images/notes/mitosis/cell_cycle_graphic.gif.

Mitosis segregates chromosomes equally between the daughter cells, resulting in genetically identical cells (Alberts et al. 2002, Campbell and Reece 2002). The ploidy is unchanged during mitosis.

Sporogenesis

In sporogenesis, the ovule primordium, containing the functional spores, and which consists of the LI, LII (reproductive layer) and the LIII layer, is formed (Koltunow and Grossniklaus 2003). The LI, LII and sometimes some cells of the LIII layer form the nucellus tissue, which later differentiates into the ovule during gametogenesis. The Megaspore Mother Cell (MMC) differentiates from the LII layer in the nucellus on the female side (Koltunow and Grossniklaus 2003), and the microspore mother cell on the male side (Figure 3). These two cells undergo meiosis, which causes the transition from the diplophasic to the haplophasic state.

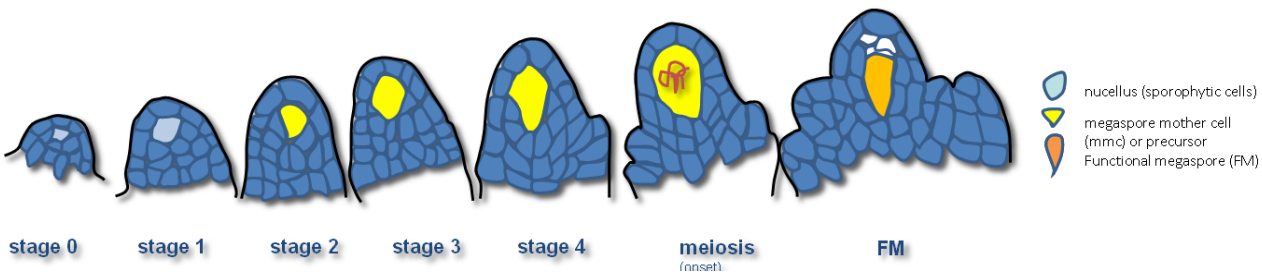


Figure 3. Sporogenesis – Transition from Diplophase to Haplophase
In sporogenesis LI, LII and LIII develop into the ovule primordium. The megaspore mother cell differentiates from the LII layer, undergoes meiosis, and one of the four meiotic products is selected to become the functional megaspore. The functional megaspore is haplophasic and initiates gametogenesis. Figure made by Celia Baroux. Used with permission.

Meiosis

Meiosis, or the reductive division, is a special cell cycle with two consecutive cell divisions, meiosis 1 and meiosis 2 (Figure 4).

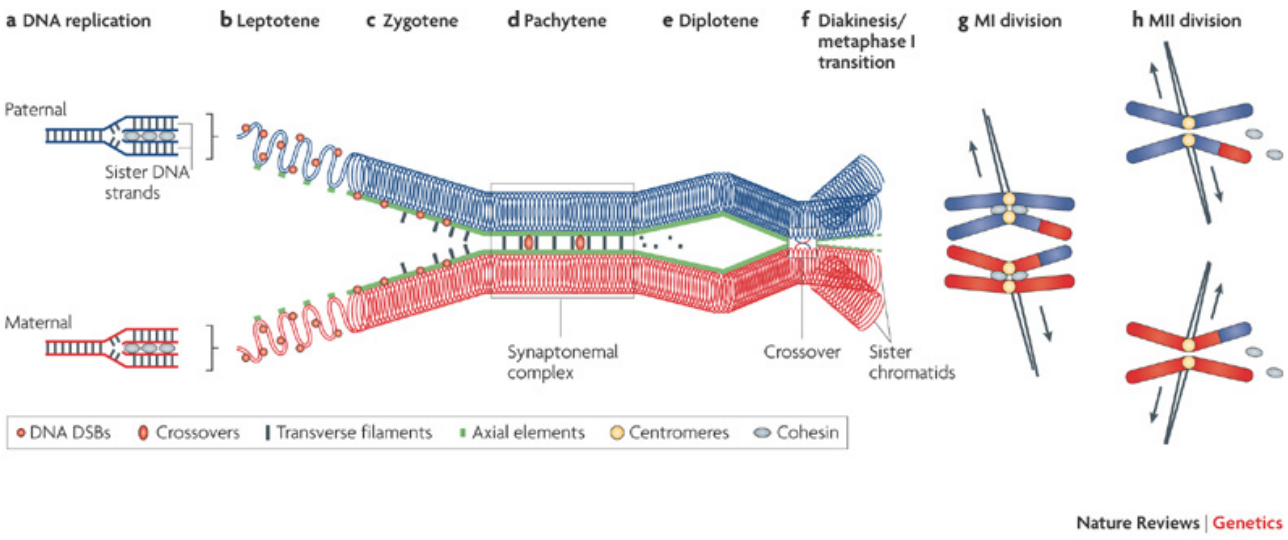


Figure 4. Meiosis – Reductional Division and Recombination
During leptotene stage double stranded breaks (red circles, DSBs) are introduced while the chromosomes condense. During zygotene the synaptonemal complex begins to form (green and blue lines) and its formation is finished in pachytene. During zygotene and pachytene, recombination occurs, resulting in crossovers. In diplotene, the synaptonemal complex disassembles and in diakinesis sister chromatids are only connected via the crossovers and cohesins around the centromere. Meiotic division 1 separates homologues, while meiotic division 2 separates the recombined sister chromatids.
blue – paternal chromosome; red – maternal chromosome; recombined chromatids are indicated by a red and blue chromatid. Figure take from (Burgoyne et al. 2009).

No DNA replication occurs between the two divisions. Prophase 1 of meiosis 1 is divided into 5 sub-phases: i) leptotene (chromosomes condense), ii) zygotene (synaptonemal complex assembles), iii) pachytene (crossovers occur), iv) diplotene (chiasmata are fully built), v) diakinesis (chromosomes condense further and are being attached to the spindle). During pachytene, recombination starts and is completed at anaphase 1 of meiosis 1, in which the homologous chromosomes are separated. Meiosis 2 is similar to mitosis, separating the sister chromatids and since no DNA-synthesis occurs between meiosis 1 and meiosis 2, ploidy is reduced. The meiotic products are four haplophasic cells.

Recombination

Recombination is the most basic process underlying sexual reproduction. The synaptonemal complex, which is fully assembled in pachytene, ensures that homologous chromosomes pair. This close pairing enables crossing over of homologous, but not identical, DNA-strands (Alberts et al. 2002). Crossovers are visible cytologically as chiasmata in diplotene (Alberts et al. 2002, Campbell and Reece 2002). In anaphase 1 of

meiosis 1, homologues are separated, and crossovers are resolved. This is different from mitosis, in which sister chromatids are separated. The result of crossovers are chromatids which are a new combination of two homologous chromatids.

Meiosis results in four haplophasic cells, each being genetically different from the others, due to recombination and segregation. These meiotic products are the basis of new genotypes, produced during sexual reproduction. At the end of sporogenesis, the functional megaspore on the female side, and the functional microspore on the male side have developed.

Gametogenesis

Unlike in animals, where the meiotic products directly differentiate into gametes, the meiotic products in plants undergo mitosis to produce multicellular gametophytes. This process is called gametogenesis (Figure 5).

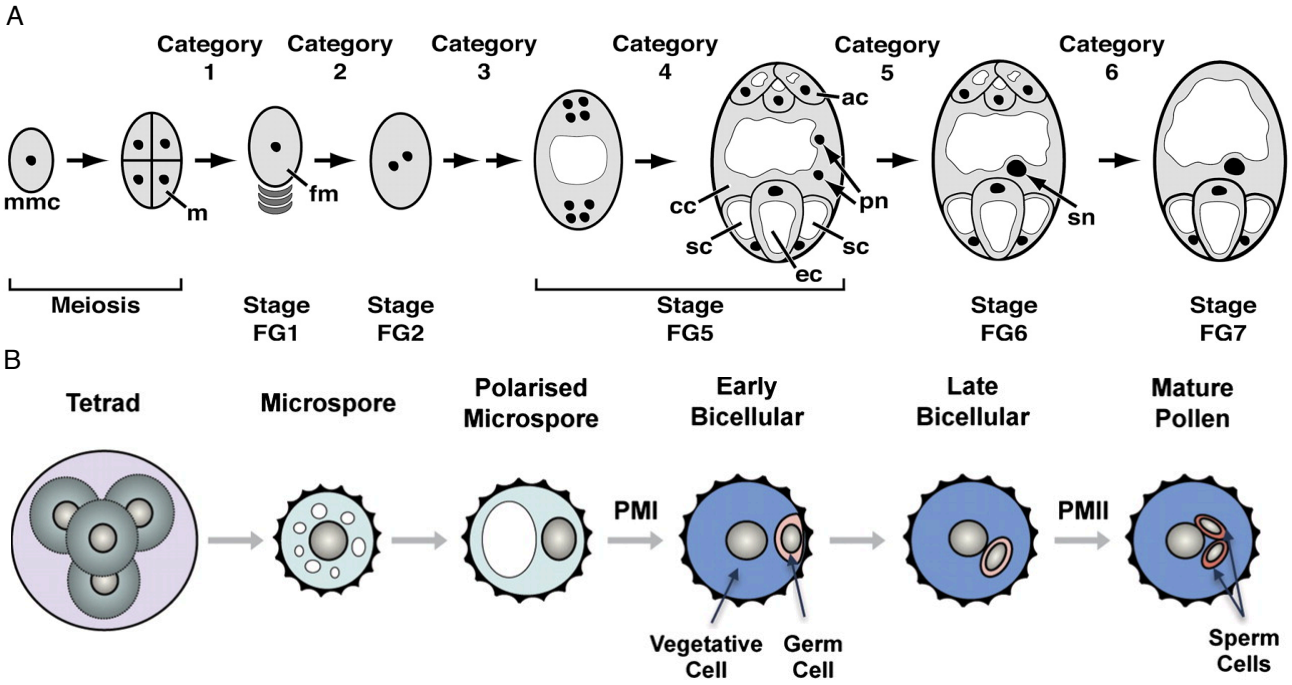


Figure 5. Gametogenesis – Development of Gametophytes

A) Female gametogenesis. After meiosis three of the haplophasic cells abort, while one develops into the functional megaspore (fm). The fm undergoes three rounds of mitosis, typically leading to an 8-nucleate, 7-celled embryo sac (polygonum type). The central cell (cc), which will develop into the endosperm after fertilization, contains two nuclei. The egg cell (ec) will develop into the embryo after fertilization. The cc together with the ec form the female gamete. Depending on the species, the two nuclei in the cc fuse before fertilization. In some species, the antipodal cells have degenerated in the mature gametophyte. mmc – megaspore mother cell; m – meiotic product; fm – functional megaspore; ac – antipodal cell; cc – central cell; sc – synergid cell; ec – egg cell. Figure taken from http://www.plantcell.org/content/16/suppl_1/S133/F3.large.jpg **B) Male gametogenesis**. The four meiotic products are called tetrad, each haplophasic cell is a microspore. The microspore undergoes two rounds of mitosis. After the first mitotic division, a bicellular pollen is formed. One cell is the vegetative cell, which contains the germ cell. The germ cell divides mitotically again resulting in two sperm cells, which are the male gametes. PMI and PMII – Pollen Mitosis I and II, respectively. Figure modified from http://jxb.oxfordjournals.org/content/60/5/1465/F1.large.jpg

Female Gametophyte

Meiosis of the MMC results in four haplophasic cells. Three of the meiotic products abort and the surviving cell is called Functional Megaspore (FM). The FM undergoes three rounds of mitosis, resulting in the 7-celled, 8-nucleate embryo sac, representing the major type of female gametophytes in flowering plants, the Polygonum type. The central cell (CC) contains two nuclei. The egg cell (EC) together with the CC constitute the female gamete (Figure 5A). Antipodal and synergid cells are called accessory cells.

Male Gametophyte

Meiosis of the microspore mother cell results in four haplophasic cells, which are the microspores. Each microspore undergoes one asymmetric mitosis after which the smaller generative cell is engulfed by the cytoplasm of the bigger vegetative cell, forming a unique cell-in-a-cell structure. The generative cell undergoes another round of mitosis, resulting in two cells, the sperm-cells or male gametes (Figure 5B). This unique 2-cells-in-a-cell structure is the male gametophyte, the pollen.

Embryogenesis

Embryogenesis is the development of the zygote to the mature embryo. It is triggered by the fusion of the female and male gametophyte to form the zygote in the process of fertilization.

Double Fertilization

In plants, one sperm cell fertilizes the EC, while the second sperm cell fertilizes the CC. Fertilization triggers mitotic cell division of both the EC to develop into the diploid embryo and the CC to develop into the triploid endosperm. Some angiosperms require a maternal to paternal genome ratio of 2:1 (2m:1p ratio) for normal endosperm development (reviewed in Haig and Westoby 1991). Deviations from this ratio can cause seed abortion, leading to a ploidy barrier (Koltunow and Grossniklaus 2003) (Figure 6).

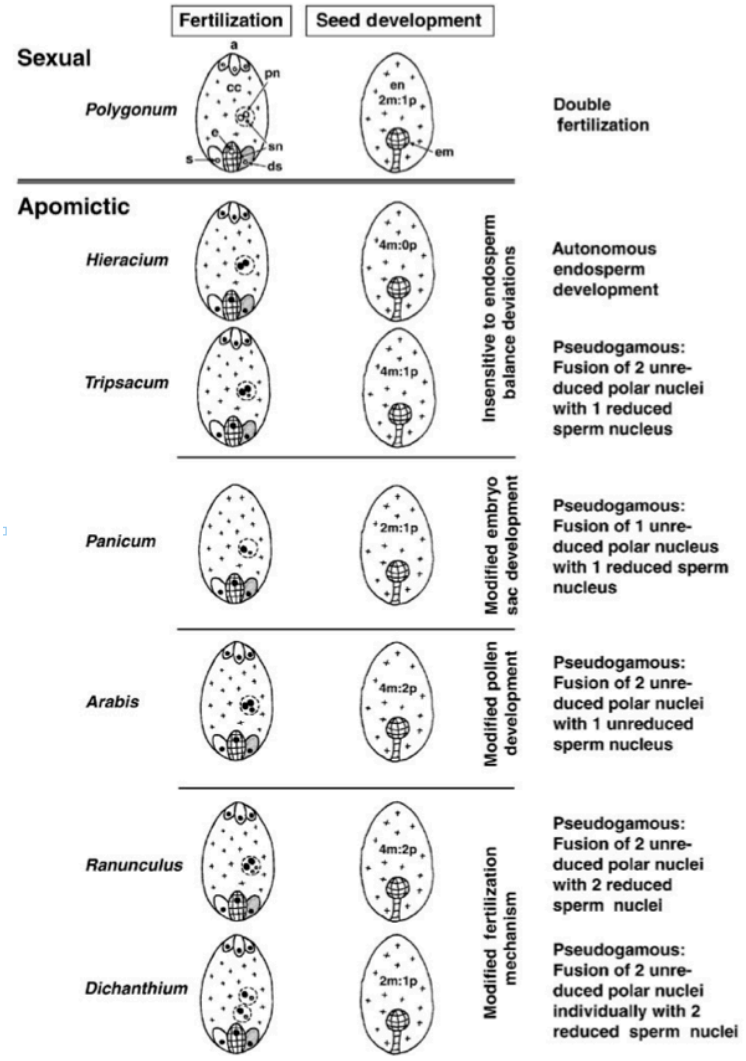


Figure 6. Ploidy Barrier – 2 Maternal : 1 Paternal Genome for Endosperm Development

In many species, endosperm development requires a maternal to paternal genome ratio of 2:1 (2m:1p). If this ratio is not achieved, the endosperm will not develop. This leads to a ploidy barrier. If there is no ploidy barrier, this ratio does not need to be fulfilled for endosperm development. Pseudogamous apomictic species have developed different mechanisms to achieve the 2m:1p genome ratio. Figure taken from (Koltunow and Grossniklaus 2003).

Apomixis

Apomixis is the asexual reproduction through seeds (Asker and Jerling 1992). To achieve this, three sexual processes have to be altered: 1) Meiosis, 2) Fertilization, and 3) Endosperm development (Figure 7).

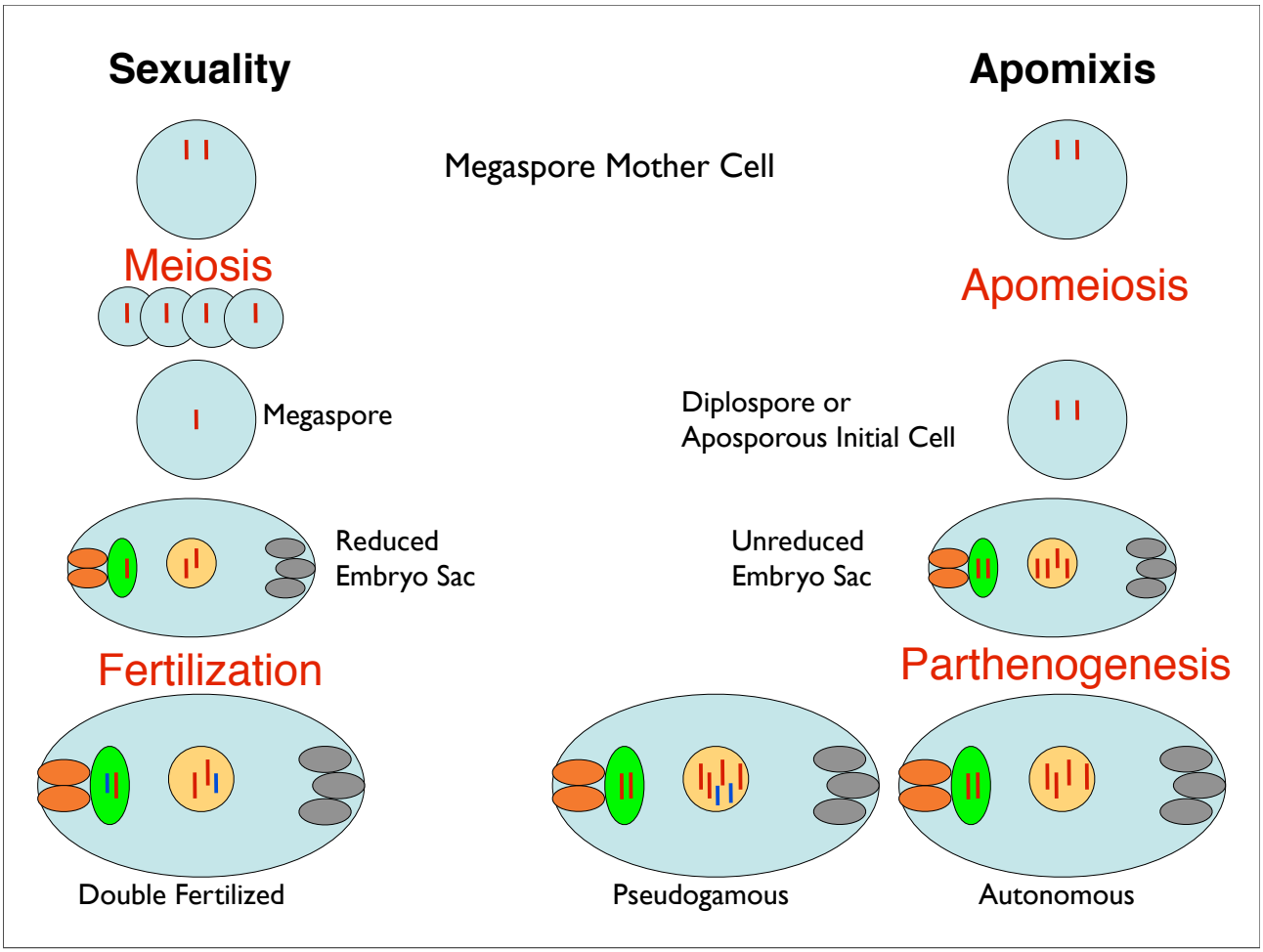


Figure 7. Apomixis
In sexuality, meiosis results in a FM, which is haplophasic and develops into a so called reduced embryo sac. Double fertilization triggers development of the embryo and the endosperm. In apomixis, the first process which is altered is meiosis. Meiosis is avoided or omitted, a process called apomeiosis. This results in a diplospore, since this cell is diplophasic. Instead of a diplospore an aposporous initial cell forms from a nucellar cell other than the MMC. Both diplo- or apospore develop into a so called unreduced embryo sac, since no reductional division occurred. The second process which is altered in apomixis is fertilization. The embryo develops without fertilization, i.e. parthenogenetically. The third altered process is the endosperm development. It can either be autonomous without fertilization, or pseudogamous, in which case the ploidy barrier has not broken down. Different mechanisms have evolved to ensure the 2m:1p genome ratio in the endosperm in pseudogamous species.

Fertilization and endosperm development happen in the female reproductive organs, the ovules. Apomixis affects female gametophyte development, while male gametophyte development is usually normal (meiosis), which enables outcrossing of apomicts via pollen.

Apomixis can be viewed as a deregulation of sexual processes in space and time (Grimanelli et al. 2001, Grossniklaus and Nogler 2001, Koltunow and Grossniklaus 2003, Ozias-Akins and van Dijk 2007), which is reflected in the facultative nature of apomixis (Asker and Jerling 1992) (Figure 7).

Sporophytic Apomixis

In sporophytic apomixis, embryos develop from a sporophytic cell. This is the case for example in *Citrus* (Asker and Jerling 1992). It is also referred to as adventitious embryony. The seed is often polyembryonic and the embryos are a mixture of a sexual and at least one apomictic embryo. Embryos of sporophytic apomictic origin are maternal clones.

Gametophytic Apomixis

In gametophytic apomixis, embryos develop from a nucellar cell (Koltunow and Grossniklaus 2003). Depending on the position of the cell which gives rise to the embryo, diplospory and apospory are distinguished.

In diplospory, the embryo develops from a cell in the position of the MMC. Due to apomeiosis, no transition from diplophase to haplophase occurs and the cell stays diplophasic, hence the name diplospory. Depending on the time-point of abortion of meiosis, mitotic and meiotic diplospory are distinguished. In case of mitotic diplospory, meiotic processes either abort very early or mitosis is entered directly (Asker and Jerling 1992, Bicknell and Koltunow 2004). Therefore, recombination cannot happen. This type of apomixis results in maternal clonal offspring. In meiotic diplospory, meiosis starts but aborts before meiosis 2 (Asker and Jerling 1992, Bicknell and Koltunow 2004). Here, recombination can still occur. This leads to so called autosegregation, as is the case for example in *Taraxacum* sp. (van der Hulst et al. 2003). Meiotic diplosporous offspring are maternal clones, however not exact copies of the maternal genomic constitution, since they can have an altered allele configuration is *cis*, due to recombination. Diplospory is a deregulation of the sexual processes in time (Koltunow and Grossniklaus 2003).

Apospory

In apospory, a so called Aposporous Initial Cell (AIC), which is not a cell in the position of the MMC, starts mitotic cell division as in gametogenesis. The sexual process occurs in parallel. In some species, the sexual product (FM) aborts, resulting in an ovule with a single, unreduced embryo sac (Koltunow et al. 1998). In other species, both sexual and apomictic embryos develop, giving rise to polyembryonic seeds, as it is also the case

in sporophytic apomixis. Since no recombination occurs in the AIC, aposporous offspring are maternal clones. Apospory is a deregulation of sexual processes in space (Koltunow and Grossniklaus 2003, Tucker et al. 2003).

Endosperm Development

Autonomous Endosperm Development

In autonomous endosperm development, endosperm develops without fertilization. This is the case in *Taraxacum* sp. and *Hieracium* sp. (Koltunow and Grossniklaus 2003). Autonomous diplospory and autonomous apospory do not require fertilization of the central cell.

Pseudogamy

In species which rely on the ploidy barrier, the endosperm needs to be fertilized in order to achieve the 2 maternal to 1 paternal genome ratio to allow normal development. Different species have modified either embryo sac development, pollen development, or the fertilization mechanism to achieve the 2m:1p ratio (Grossniklaus et al. 1998, Grossniklaus and Nogler 2001, Koltunow and Grossniklaus 2003) (Figure 6).

Economic Value of Apomixis

Apomixis fixes genomic constitutions by clonal reproduction, except for some offspring generated by meiotic diplospory. It is therefore thought that apomixis could fix hybrid vigor (heterosis). Heterosis is the greater vigor of growth, survival, and fertility in hybrids than in the parents (Chen 2010). Fixed hybrid vigor would be economically beneficial in plant breeding, since the yearly crosses to produce heterotic hybrids would only have to be done once (Spillane et al. 2001). Apomictic reproduction would enable the indefinite reproduction of desired heterotic hybrid lines (Spillane et al. 2001, 2004).

Ecological and Evolutionary Implications of Apomixis

Sexuality results in offspring which are genetically diverse. New genotypes are produced in every generation by shuffling existing variation, which has accumulated in the population through mutations. Variation is the basis of evolution (Darwin 1861). In asexual reproduction as in apomixis the genotypes are fixed. Offspring will carry all alleles of the mother, even in the case of meiotic diplospory in which the allele configuration changes in *cis* due to recombination. In a hypothetical obligate apomictic population, genotypic variation will be fixed, while in an obligate sexual population, genotypic variation will change each generation. Apomicts can still outcross and their facultative nature enables generation of new genotypes, but apomicts have a limited scope for recombination and, therefore, generating less combinations of genetic variation, compared to sexuals (Darlington 1958). This limitation in generating variation led Darlington to conclude that apomixis is a blind alley of evolution, leading ultimately to the extinction of an apomictic species (Darlington 1958). Still, apomixis is found in over 400 plant species (Asker and Jerling 1992), suggesting evolutionary benefits of asexual reproduction.

Reproductive Assurance

Considering a monocarpous (flowering once per life cycle, Lincoln et al. 1998), obligate outcrossing species, finding a mate is essential to reproduce. In sparse population densities of such a species, as they might occur after long distance dispersal, reproductive assurance is of vital importance.

One possibility to ensure reproduction in such species would be the breakdown of self-incompatibility. Tomlinson (1966) modeled the chance of finding a mate, and therefore successful reproduction, as a function of individuals in the effective breeding area (population density) and the mode of reproduction. Selfing species have reproductive assurance, since they do not need to find a mate to reproduce (Figure 8).

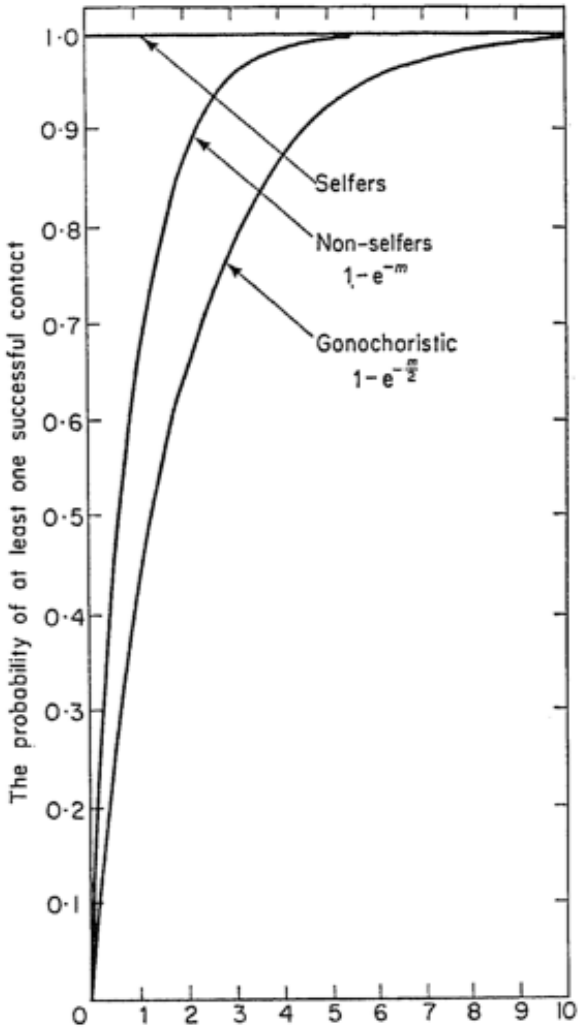


Figure 8. The Chance of Finding a Mate
The chance of finding a mate follows a poisson distribution. The probabilities depend on the mode of reproduction. The x-axis indicates the number of possible mates within the effective breeding area (the area which gametes can travel), the y-axis represents the probability of a successful breeding contact. Non-selfers – hermaphroditic organisms; Gonochoristic – different sexes are in different individuals. Figure taken and modified from (Tomlinson 1966).

Baker (1955, 1967) has taken this thought further based on observations of colonization of remote islands. Baker's law states that a single selfing individual is enough to found a new population (Baker 1955), even though this might be a very rare event, due to demographic impacts.

Apomixis – Escape from Sterility

The considerations for selfing species are also true for apomictic species. Apomicts do not need to find a mate to reproduce, and a single individual is sufficient to found a new population. In other words, apomixis provides the same reproductive assurance as selfing does. However, selfing does not ensure reproduction in case of odd ploidy levels. Odd ploidy levels result in disturbed meiosis, leading to abortion of meiosis or meiotic products. In other words, individuals with odd ploidy are sterile. Apomeiosis circumvents this problem. Therefore, apomixis does not only provide reproductive assurance in the absence of a mate, but additionally provides an escape from sterility (Darlington 1958).

Muller's Ratchet

Despite the ecological similarities between selfing and apomixis, a main difference between these two remains. Repeated selfing results in isogenic lines, or in other words, homozygosity at all loci. Apomixis on the other hand fixes heterozygosity. For the following arguments, only large populations sizes are considered, which makes genetic drift negligible for this discussion.

Mutations constantly arise, either by environmental influences or by biotic influences, as for example mistakes in DNA-replication during S-phase (Alberts et al. 2002). Mutations usually arise in a heterozygous state (Muller 1964, Kondrashov 1985). Some of these mutations can be advantageous, but most mutations will have a negative effect on fitness. Muller (1932) acknowledged recombination as the mechanism to shuffle and distribute these mutations, but the main function of recombination is the generation of new genotypes. Despite this distributive (segregation) function of recombination, it is a mechanism to remove deleterious mutations from the genome (Muller 1964). An asexually reproducing species does not have this possibility, since it avoids recombination. Therefore, mutations accumulate over generations, leading to the extinction of certain genotypes, the mutational load of which has reached a critical number (Muller 1964, Kondrashov 1985). The mutational load cannot be diminished in asexual genotypes, it is an irreversible process, such as the progressive movement of a ratchet.

Selfing, and therefore sexual, species are following Muller's ratchet as much as asexual species do, since they have lost the evolutionary advantage of recombination (Muller 1964). Repeated selfing leads to inbreeding depression, which is the reduction of fitness and vigor by increased homozygosity, as a result of inbreeding in a normally outbreeding population (Lincoln et al. 1998). Inbreeding depression might be due to the expression of deleterious mutations in homozygotes (Lande and Schemske 1985). Muller's ratchet is therefore a possible explanation for inbreeding depression. The removal of deleterious mutations from the genome due to recombination comes at the cost of producing homozygous mutant genomes in the same process. This becomes more likely with the degree of inbreeding and therefore with the level of homozygosity. However, due to possible outcrossing in selfing species, the mutational load could be decreased by recombination, which, in outcrossing, segregates genotypes which are free of the mutations, which in turn can be selected (Muller 1964). This line of thought is also true for apomixis, which is a deregulation of sexual processes in space and time (Koltunow and

Grossniklaus 2003, Tucker et al. 2003), and therefore of facultative nature (Asker and Jerling 1992), enabling the generation of new genotypes by outcrossing.

Geographical Parthenogenesis

Despite the theoretical evolutionary caveats of apomixis, apomictic cytotypes are geographically more widespread than sexual cytotypes (van Dijk 2003, Mráz et al. 2008, Hörandl 2009, Cosendai and Hörandl 2010, Figure 9).

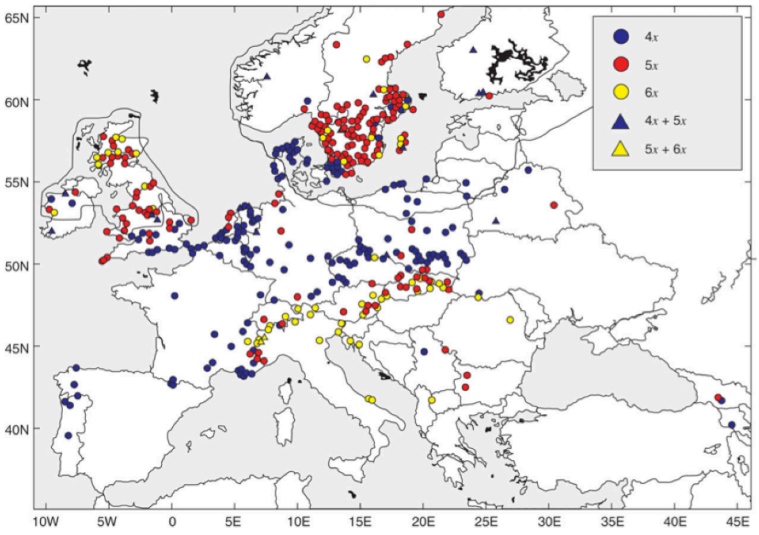


Figure 9. Geographical Parthenogenesis – Apomicts Are Geographically More Widespread
The example given here is from *Hieracium pilosella* L.. Pentaploid cytotypes (red circles) are apomictic and geographically more widespread than the sexual tetraploid cytotypes (blue circles). Triangles refer to populations consisting of several ploidy levels. Figure taken from (Mráz et al. 2008).

Three hypotheses explaining this pattern have been formulated. All hypotheses acknowledge genetic diversity of clones.

Destabilizing Hybridization

Lynch (1984) has formulated the destabilizing hybridization hypothesis. It states that a successful genotype is destabilized by hybridization, since hybridization (inter- and intraspecific) leads to the destruction of successful allele combinations. Generation of destabilized hybrids by crossing with sexuals is reduced in marginal habitats due to the prevalence of apomicts, since they have reproductive assurance.

General Purpose Genotype

This hypothesis was originally formulated by Baker (1965) and used by Lynch (1984) to explain geographical parthenogenesis. It states that selection will favor clones which are adapted to a wide variety of environments. In other words, general-purpose genotypes are selected. Sexuals fail to spread to marginal habitats since their fitness will be inferior to the fitness of the general purpose genotypes occurring there (Lynch 1984). The general purpose genotype would correspond to the heterosis model of niche occupation as described in Vrijenhoek (1984, Figure 10).

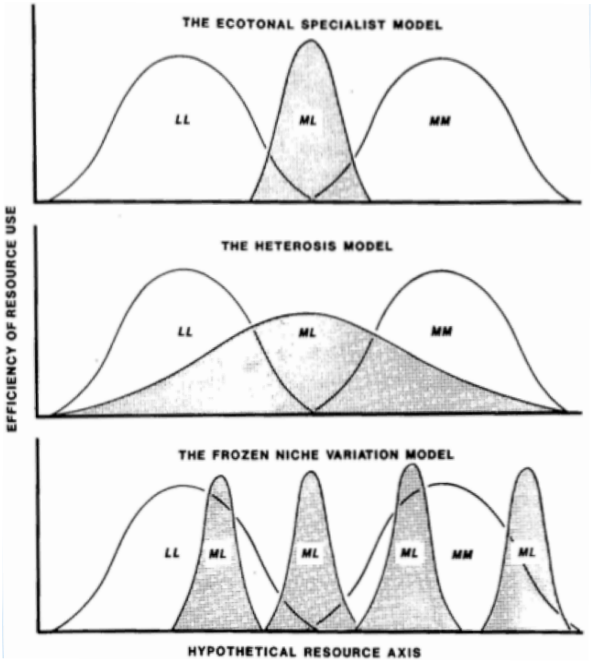


Figure 10. Frozen Niche Variation – Different Clones Occupy Different Small Niches

This figure represents different possible niche occupations for hybrids (ML) of the two *Poeciliopsis* species (MM & LL). The middle graph would correspond to a general purpose genotype. The bottom graph represents the frozen niche variation in which different clones occupy different small niches (frozen niches) which overlap the niches of the parents. The sum of the frozen niches can cover a bigger niche width than the niches of the parents. Figure taken from (Vrijenhoek 1984).

Frozen Niche Variation

Vrijenhoek (1984) formulated the Frozen Niche Variation hypothesis based on observations in triploid fish of the genus *Poeciliopsis* (Vrijenhoek 1979). Hybridization of two sexual species leads to new genotypes which are specialists and occupy a narrow niche (Figure 10). In the hybridization process, several narrowly adapted genotypes are generated. Interclonal selection leads to occupation of different small niches, leading to coexistence of clones that are ecologically different. Asexual reproduction would freeze these various narrowly adapted genotypes, which are occupying various small niches. The ecological variation of all frozen niches together could cover a bigger ecological niche than the one of the sexual ancestors (Vrijenhoek 1984). This would explain the wider spread of apomicts compared to sexuals.

Invasion

Invasion is the expansion of a species into an area not previously occupied by it (Booth 2003). Catford et al. (2009) have summarized different models of invasion into the PAB (Propagule pressure, Abiotic characteristics, Biotic characteristics) model (Figure 11), and divided the invasion process into 6 stages: 1) Transport, 2) Introduction, 3) Colonization, 4) Naturalization, 5) Spread, and 6) Impact.

Stage/Process	1/Transport	2/Introduction	3/Colonization	4/Naturalization	5/Spread	6/Impact*
Definition	Movement of plants or plant propagules to new location	Arrival of plant or plant propagules into new location	Survival of introduced plants	Survival and reproduction enabling pioneer population to be self-sustaining	Dispersal of propagules and spread of populations outside of area where first introduced	Harmful impact of species to ecology and economy
Driving factor†	P	P	PAB	pAB	PAB	paB
Spatial scale‡	Regional and continental	Local	Local	Local	Regional	Local and regional
Human-assisted	Yes, generally	Yes, generally	Yes, but not essential	No	No, but can exacerbate	No
Potential management actions	Quarantine and screening	Monitoring, detection and early eradication	Monitoring, detection and early eradication	Eradication and control of founding population; control of potential dispersal vectors	Dispersal and spread minimization; detection and eradication of satellite populations	Population control; dispersal and spread minimization; impact alleviation

Figure 11. The 6-stage Invasion Process
This table summarizes the different stages of invasion. Table taken and modified from (Catford et al. 2009).

Different combinations of P, A and B of the invading species can explain the different stages of invasion (Catford et al. 2009). Selection of genotypes occurs at each stage, whereupon transportation and introduction can be seen as a bottleneck (Nei et al. 1975).

Apomixis is a trait adding to propagule pressure due to its reproductive assurance, and it is a biotic characteristic. It is therefore promoting all six steps in the invasive process. Other traits, despite apomixis, which are advantageous biotic characteristics, can be fixed and reproduced via apomixis. Sexual species, heterozygous for advantageous traits, would either segregate these traits or could enter an inbreeding depression if they are selfing. Apomixis is therefore a trait that is advantageous in invasion, if the founding population successfully passed a bottleneck and genetic drift.

Succession

Ecological succession is the gradual and predictable process of progressive community change and replacement, leading towards a stable climax community (Lincoln et al. 1998).

Primary succession refers to an ecological succession commencing in a habitat or on a substrate that has never previously been inhabited (Lincoln et al. 1998). Primary succession can be seen as a form of invasion, since species expand into a previously unoccupied area. All aspects of the PAB-model and the 6 stages of invasion described above, apply here as well.

Competition as a Mechanism of Selection

Competition is the simultaneous demand by two or more organisms or species for an essential common resource that is actually or potentially in limited supply (exploitation competition), or the detrimental interaction between two or more organisms or species seeking a common resource that is not limiting (interference competition) (Lincoln et al. 1998).

During invasion, as well as during succession, species and individuals have to compete for available resources. Successfully competing genotypes will be able to reproduce, which is an essential step during the colonization and naturalization stages in invasion. Genotypes that are not successfully competing will go extinct. In an evolutionary sense, competition applies selection pressure. Competition itself is the sum of habitat variations and species communities. Outcrossing species are thought to have an advantage, since new genotypes are generated each generation, possibly producing well adapted genotypes (Stebbins 1950). On the other hand, segregation leads to destruction of these genotypes (destabilizing hybridization, Lynch 1984). Asexual reproduction, either via stolons or apomixis, would fix a successful genotype. Selection acts on these successful genotypes, which can result in extinction due to environmental changes, or in establishing a frozen niche (frozen niche variation, Vrijenhoek 1984). Apomixis supplies reproductive assurance of successful genotypes (Baker 1955, Darlington 1958, Tomlinson 1966), but it still enables outcrossing and generation of new genotypes (Asker and Jerling 1992, Koltunow and Grossniklaus 2003). Stebbins (1957) considered such a species as successfully adaptable, meaning that this species can cope with a variety of possible events and not go extinct.

Hieracium pilosella L. – Model Organism to Investigate Evolutionary and Ecological Aspects of Different Modes of Reproduction

The diversity of topics and questions addressed in this thesis, ranging from mode of reproduction to evolutionary fitness, require a model organism which is capable of all three modes of reproduction.

Hieracium pilosella L. is a monocarpous, herbaceous, autonomous aposporous apomict. Monocarpous means that the plant flowers once per life cycle. Autonomous apospory is apospory with autonomous endosperm development. This means that apomictic reproduction does not require fertilization at all, and leads to apomictic offspring which are maternal clones with the exact genomic constitution as the mother. Additionally, *H. pilosella* can also reproduce vegetatively via aboveground stolons. Vegetative reproduction enables clonal propagation of sexual and apomictic lines alike, and the same reproductive generation of the lines is kept. Furthermore, different ploidy levels ranging from 3C to 8C (1C is the haploid genome, Greilhuber et al. 2005, Mráz et al. 2008) are observed in natural populations and each ploidy level can consist of sexual and apomictic lineages (Mráz et al. 2008, Krahulcová and Krahulec 2011). Plants of different ploidy can be successfully crossed, since there is no ploidy barrier in this species.

Since apomixis in *H. pilosella* does not require fertilization, plants can be tested for apomixis via decapitation (Koltunow et al. 1995). In decapitation, the top of the capitulum is cut off before it opens, thereby removing anthers and style, which prevents possible pollination. Apomictic plants will have a mixture of failed seed and apomictic seed set, while sexual plants will only have failed seed set (Figure 12). Decapitation ensures apomictic reproduction in experiments.

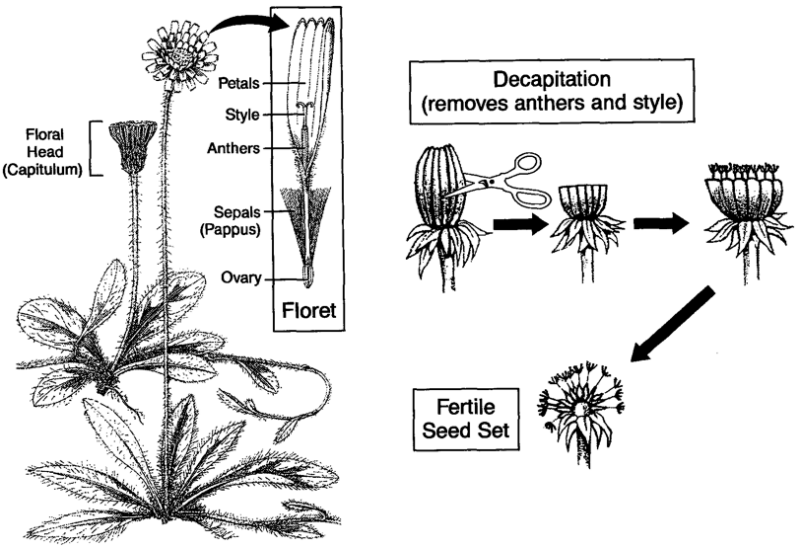


Figure 12. Decapitation Ensures Apomictic Reproduction
Cutting off the top of the capitulum before it opens removes anthers and stigma. This prevents pollination. Mature seeds therefore have to be of apomictic origin. The decapitation is only possible in autonomous apomicts. Figure taken from (Koltunow et al. 1995).

Cytological and Molecular Characteristics of Apomixis in *Hieracium* spp.

Deletion mapping in the apomictic species *H. caespitosum* (a related species to *H. pilosella*) identified two loci responsible for the apomixis phenotype (Catanach et al. 2006). One locus is responsible for apomeiosis (*Loss of Apomeiosis 1*, *LOA1*) and one locus is responsible for parthenogenesis (*Loss of Parthenogenesis 1*, *LOP1*).

In *H. pilosella,* the FM aborts if the AIC starts gametogenesis (Koltunow et al. 1998). *LOA1* has been shown to be required for initiation of apomixis, by being required for AIC formation (Okada et al. 2007). Further analysis of the different deletion lines revealed that sexual reproduction is the default reproductive pathway (Koltunow et al. 2011). The close interrelation of sexuality and apomixis was also shown by Tucker (2003) using reproductive tissue marker lines. Tucker (2003) has shown that genes necessary for embryo and endosperm development are expressed in both apomicts and sexuals. Furthermore, the two-locus model explains occurrence of the four different possible offspring types of an apomictic individual. The four different types (following the nomenclature of Harlan and deWet 1975) are: 1) n + 0, also referred to as polyhaploids, which are meiotic and parthenogenetic, 2) n + n, sexual offspring, sometimes referred to as B_{II}-hybrids, 3) 2n + 0, also referred to as maternal clones, which are apomeiotic and parthenogenetic, and 4) 2n + n, also referred to as B_{III}-hybrids, which are apomeiotic and fertilized (Rutishauser 1969, Bicknell et al. 2003) (Figure 13).

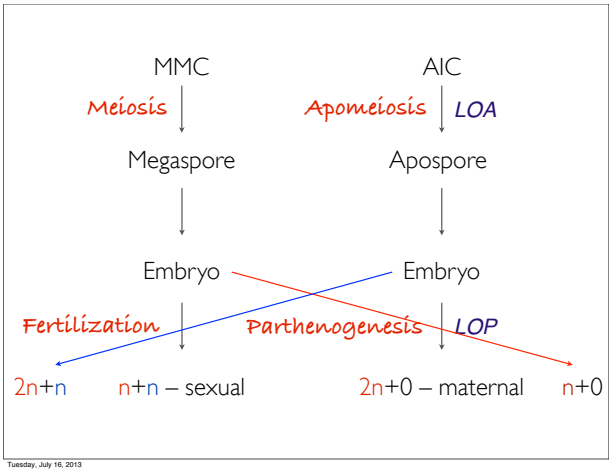


Figure 13. Four Different Types of Offspring in a Facultative Apomict
Apomeiosis and parthenogenesis are encoded on two different loci, LOA1 and LOP1. In the case of absence of both, sexual reproduction happens, leading to n + n offspring. If both loci are present, apomictic reproduction happens leading to maternal clonal offspring 2n + 0. If apomeiosis and fertilization occur, ploidy is increased. The offspring is referred to as BIII-hybrid or 2n + n. In case of meiosis and parthenogenesis, the ploidy of the offspring is halved. They are referred to as polyhaploids or n + 0. If the mother is a facultative apomict, all 4 types of offspring can occur on a single individual.

H. pilosella L. offers the possibility to compare apomixis and sexuality and also different ploidy levels. It is an ideal model system to test ecological and evolutionary hypotheses about the effects of different modes of reproduction.

Aim of Thesis

As mentioned above, reproduction by selfing and by apomixis pose ecological similarities, such as reproductive assurance and hence a conditional advantage in sparse population densities. However, they differ in terms of their evolutionary potential due to the avoidance of meiosis during female sporogenesis. Most experiments to date have been of theoretical nature or performed with selfing species.

Here, I address three main questions: 1) Does apomixis confer a conditional advantage/reproductive assurance, 2) Can complex phenotypes be fixed by apomixis, and 3) Do apomictic species have a low evolutionary potential?

Addressing the first question involved competition experiments in the common garden with lines from New Zealand and Europe (chapter 1), and with apomictic and sexual siblings (chapter 2). To cover a longer time period I performed an observational field study along a primary successive gradient in the Swiss Alps (chapter 3). Addressing the second question involved the generation of new apomictic lineages and propagating them apomictically for several generations with phenotyping each generation, a work which is still in progress (chapter 4). To address the third question all data from all experiments are integrated in the general discussion of this thesis.

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Sexual Hieracium pilosella plants are better between-species, while apomictic plants are better within-species competitors

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Abstract

Apomixis, asexual propagation through seeds, is known in over 40 plant families. This widespread phenomenon can lead to the fixation of successful genotypes, resulting in a fitness advantage. On the other hand, apomicts are expected to lose their fitness advantage if the environment changes because of their limited evolutionary potential due to low genetic variability and because of the potential accumulation of deleterious somatic mutations over many generations. Nonetheless, some apomicts have been extremely successful, for example certain apomictic accessions of *Hieracium pilosella* L. from New Zealand, where the plant is invasive. Here, we investigate whether the success of these apomictic accessions could be due to a fitness advantage by comparing the vegetative competitiveness of apomictic *H. pilosella* from New Zealand with sexual accessions of *H. pilosella* from Europe. Sexual and apomictic plants were grown either (i) alone (no competition), (ii) in competition with the other type (intra-specific competition), and/or (iii/iv) in competition with the grass *Bromus erectus* (inter-specific competition/intra- and inter-specific competition). We show that sexual plants are better inter-specific competitors than apomicts in terms of vegetative reproduction (number of stolons) and vegetative spread (stolon length), while apomicts do better than sexuals in intra-specific competition. The magnitude of the effect was in some cases dependent on the ploidy levels of the plants. Furthermore, apomicts always produced more stolons than sexuals, suggesting potential displacement of sexuals by apomicts where they co-occur.

Key words: apomixis; sexuality; inter-specific competition; intra-specific competition; invasiveness

Introduction

Apomixis, defined as asexual reproduction through seeds, is reported in over 400 species from more than 40 plant families (Asker and Jerling, 1992). Apomixis is divided into sporophytic (embryos develop from sporophytic tissue) and gametophytic apomixis (embryos develop from gametophytic tissue), the latter being further subdivided into apospory and diplospory, depending on the cell type which develops into the female gametophyte (Koltunow and Grossniklaus, 2003). Both gametophytic types can be autonomous (neither fertilization of central cell nor egg cell to form the endosperm and embryo, respectively) or pseudogamous (fertilization of the central cell, but not of the egg cell). All types of gametophytic apomixis are facultative with varying levels of sexuality (Asker and Jerling, 1992). Frequently, apomictic and sexual lineages co-occur in apomictic species (eg. Taraxacum and Chondrilla species, van Dijk, 2003; Ranunculus species, Hörandl and Paun, 2007; Hieracium pilosella, Mráz et al., 2008), with apomictic lineages being more widespread than sexual ones (van Dijk, 2003; Hörandl, 2006; Hörandl and Paun, 2007; Mráz et al., 2008).

Because apomictic plants can produce offspring through seeds without the need of finding mates (no cost of sex, Smith, 1978), they are thought to have an advantage in colonizing habitats after disturbances and as pioneer plants in succession (Tomlinson, 1966). The colonizing ability of apomicts (Baker, 1967; Smith, 1978) is thought to be advantageous in invasive processes (Baker, 1967; Catford et al., 2009). Indeed, apomictic lineages are found to be among the first to invade new areas (eg. Krahulcová and Krahulec, 2011). Based on these arguments, apomicts are often considered to be more successful than sexuals.

However, most types of apomixis result in maternal clonal offspring (apospory, mitotic diplospory) (Asker and Jerling, 1992) and may therefore accumulate deleterious mutations over many generations (Muller's ratchet, Muller, 1932; Stebbins, 1950; Stebbins, 1957). Furthermore, apomictic populations are expected to have very low genetic variability, which drastically reduces their evolutionary potential to adapt to environmental changes. Sexual populations, in contrast, are expected to have high genetic variability and can, therefore, more easily adapt to environmental changes via evolutionary processes. Hence, the question arises whether the high abundance of apomicts is restricted to newly colonized habitats or if they may successfully co-exist with sexuals due to other advantages than those mentioned above.

Several hypotheses have been proposed in this context. For example, successful apomicts may contain general-purpose-genotypes (Baker, 1965; Lynch, 1984), avoid the cost of sex (Smith, 1978), have a frozen niche variation that allows them to exploit heterogeneous habitats (Vrijenhoek, 1979; Vrijenhoek, 1984), or benefit from positive genetic diversity effects (cf. eg. Schmid and Dolt, 1994; Crutsinger, 2006). As mentioned above, all types of gametophytic apomixis have residual sexuality. Furthermore, meiotic diplospory can result in auto-segregation, as is the case in *Taraxacum officinale* (van der Hulst et al., 2003). In this type of apomixis, recombination but no reduction and fertilization occurs, leading to novel genetic configurations although the allelic composition is identical to that of the mother plant (Asker and Jerling, 1992). In addition, since usually only the female gametophyte is affected by apomixis, populations of apomictic plants have a certain potential for adaptation via sexually produced genotypes. Indeed, van der Hulst and colleagues (2003) have found that the genetic variability in an apomictic population of *Taraxacum sp*. is as high as expected for a sexual species, which is mainly explained by hybridization with co-occurring sexual lineages and/or auto-segregation. Empirical data suggest that varying degrees of sexuality play the most important role for diversification in apomicts (Hörandl and Paun, 2007).

Hieracium subgenus *Pilosella* is an example for an extremely successful invasive species complex including sexual and apomictic lineages. Sexual lineages of *H. pilosella* L. are obligate out-crossers and self-incompatible. Apomictic lineages of *H. pilosella* are aposporous apomicts with autonomous endosperm development (Koltunow et al., 1998). Apomicts occur at ploidy levels from 4C to 8C (1C = haploid genome) (Greilhuber et al., 2005; Mráz et al., 2008) with varying levels of sexuality. Aposporous apomictic offspring are true maternal clones without chromosomal rearrangements, meaning that the offspring inherit the unchanged maternal genotype. Using *H. pilosella* as a model in ecological experiments thus avoids the confounding effects of auto-segregation and of ploidy effects. In addition, population maintenance in *H. pilosella* is possible by vegetative propagation via aboveground stolons for both sexual and apomictic lineages (Bishop and Davy, 1978). Using a grid-based simulation model, Winkler and Stöcklin (2002) have shown that most rosettes of *H. pilosella* are of vegetative origin, which enables sexual and apomictic lineages to persist over many vegetation periods.

H. pilosella was accidentally introduced from Europe to New Zealand in the 1850s (Murphy, 1878), where, after a lag-phase, it became invasive and spread enormously since the 1950s (Scott et al., 1990; Connor, 1992; Houliston and Chapman, 2004). During the lag-phase of invasion, hybridizations with another introduced apomict of the subgenus

Pilosella, *Hieracium praealtum*, occurred at least 3 times and led to new genotypes (Morgan-Richards et al., 2004; Trewick et al., 2004). It can be assumed that these genotypes were selected for traits promoting reproduction and spreading, such as apomictic seed production or a high vegetative reproduction rate via stolons. It has been shown that dense seed rain and high seedling survival largely contribute to the invasive success of *H. pilosella* in New Zealand (Makepeace, 1985; reviewed in Day and Buckley, 2010). Today, this species is particularly widespread in disturbed short-tussock grassland dominated by *Festuca novae-zelandiae* (Makepeace, 1985; reviewed in Day and Buckley, 2010). The predominant cytotype occurring in New Zealand is an apomictic pentaploid (aP5, apomictic Pilosella 5-ploid, Chapman et al., 2000). In contrast, the main type occurring in Europe is a sexual tetraploid (sP4, Mráz et al., 2008).

In the present study we compare the performance of apomictic lineages from New Zealand with sexual lineages from central Europe. We took apomictic plants from New Zealand for 2 reasons: First, we can assume that due to hybridizations and different selection regimes for the past 130 years, New Zealand and European lineages of *H. pilosella* diverged genetically from each other in terms of their population dynamics. Second, apomictic plants from New Zealand have a high level of apomixis (Bicknell et al., 2003; Bicknell and Koltunow, 2004; Houliston and Chapman, 2004). We expected that the sexual lineages from Europe have been selected for competitiveness and persistence of populations and the apomictic lineages from New Zealand for high vegetative reproduction and spreading.

We carried out two experiments. First, in a common garden experiment, we compared offspring of *H. pilosella* plants from New Zealand with offspring of sexual populations from Europe for their inter-specific (between-species) and intra-specific (within-species) competitiveness. Second, we tested whether differences in competitiveness between sexual and apomictic lineages persisted when ploidy was equal. To do this, we compared the apomictic pentaploid lineages from New Zealand (aP5) with newly created sexual pentaploid lineages (sP5). Pentaploid sexuals were obtained by crossing of sexual hexaploid (sP6) and sexual tetraploid (sP4) plants. By comparing the two independent experiments, we can draw conclusions about the role of ploidy for the performance of *H. pilosella*.

In both experiments, fitness-related traits (biomass, number of stolons, length of the longest stolon) were measured and the effects of reproductive type and intra- vs. inter-specific competition were tested using mixed-effects models.

We found that the sexual lineages from European populations were more persistent in between-species competition against the grass *Bromus erectus* in terms of vegetative reproduction, irrespective of ploidy level or genetic background. However, when apomictic lineages from New Zealand grew in competition with sexual lineages from Europe they had a superior fitness, suggesting that they can displace the sexual lineages in within-species competition.

Materials and Methods

Plant Materials and Soil

Hieracium pilosella L. (syn. *Pilosella officinarum* Schultz & Schultz) is a self-incompatible perennial monocarpic herbaceous species including sexual and apomictic lineages (autonomous apospory) with different ploidy levels. Plants can reproduce vegetatively via aboveground stolons.

Sexual lineages were derived from two sexual tetraploid populations in the Czech Republic. One population was from Mšeno (M; N 50° 28´17.5´´, E 14° 38´1.2´´) the other from Jince (J; N 49° 46´45.7´´, E 13° 57´54.4´´). Seeds were kindly sent to Zürich by Anna Krahulcova (Academy of Sciences of the Czech Republic). Apomictic lineages were derived from an apomictic population in New Zealand at Hurunui River near Lake Sumner (S 42° 42', E 172° 08'). Seeds were kindly collected by Ross A. Bicknell (Plant & Food Research, New Zealand) in March 2009. Apomictic plants used in the experiments were grown from seeds of several individuals from the New Zealand population and consist of a mixture of different apomictic pentaploid lineages (aP5). Sexual plants were derived from vegetatively propagated plants from the two Czech populations (M; J), and are a mixture of 3 sexual tetraploid lineages (sP4). To generate pentaploid sexual lineages (sP5), a sexual tetraploid (sP4, from Jince) and several sexual hexaploid lineages (sP6) from the Morteratsch glacier foreland, Upper Engadin, Switzerland (GPS: 791859, 145592, Swiss Grid) were crossed.

After surface sterilization, seeds were germinated in petri dishes on half strength MS-medium (Murashige and Skoog, 1962) (containing MS salts (Carolina, Burlington, North Carolina), Sucrose (Applichem, Darmstadt, Germany) and Phytoagar (Gibco BRL, Paisley, Scotland)) in a Percival Scientific climatic cabinet (CU-36L6/D, CLF Plant Climatics GmbH, Wertingen, Germany) at 22°C/18°C (day/night) 14h light and 10h dark cycle. Seedlings were transferred to soil when they had produced two to three true leaves. Seedlings were grown in the greenhouse for three days under a humidor after being transferred to soil, and then put into the common garden or left in the greenhouse. We used a nutrient-poor soil ("Dachgartenerde extensiv", Ricoter Erdaufbereitungs AG, Frauenfeld, Switzerland) to mimic the field situation.

Experimental Design

Two experiments with 3 fully crossed treatments were performed (Fig. 1A), growing apomictic and sexual plants (2 levels of reproduction), either alone or in competition with

the other reproductive type (within-species competition), and with or without competition with the grass *Bromus erectus* Huds. (between-species competition). We used *B. erectus* to create a similar novel competitive environment for both apomictic and sexual lineages. This resulted in 4 different treatment combinations for both reproductive types: 1) no competition (Fig. 1B i), 2) between-species competition (presence of grass, Fig. 1B iii), 3) within-species competition (different reproductive mode of neighbor, Fig. 1B ii), and 4) both between- and within-species competition (Fig. 1B iv).

In the first experiment ("garden") apomictic and sexual plants of *H. pilosella* were derived from field sites and were of different ploidy level. This experiment was performed in the common garden with several apomictic lineages from New Zealand and three sexual lineages from Europe.

In the second experiment ("P5"), apomictic lineages from the New Zealand population were used together with the created sexual pentaploid lineages to ensure that apomictic and sexual plants were of the same ploidy level. Both apomictic and sexual plants were grown from seeds. This experiment was performed in the greenhouse.

In each experiment, plants were grown in plastic boxes (Georg Utz AG, Bremgarten, Switzerland) of 40 x 30 x 30 cm (L x W x D). The bottom of the boxes had holes and was covered with a 2-cm thick drainage mat to prevent root rotting in standing water. The boxes were covered with mosquito net (Windhager AG, Baar, Switzerland) cages to prevent pollination between experimental units in the common garden. Boxes with a cage were considered as experimental units. In each box, positions of sexual and apomictic *H. pilosella* plants were fixed in a grid of 12 positions for the garden experiment and 8 positions for the P5 experiment. Individuals were randomly assigned to these positions (Fig. 1B i,ii). For between-species competition (Fig. 1B iii, iv), 6 *H. pilosella* plants were grown alternating with 6 plants of the grass (*B. erectus* Huds. from the Swiss Jura Mountains; Otto Hauenstein Samen, Rafz, Switzerland). For within-species competition, sexual and apomictic plants were planted alternately in a ratio of 1:1 (Fig. 1B ii). To control for position effects, the order was switched in every second experimental unit. For the P5 experiment, 4 plants were used instead of 6 to give them more space for stolons.

In the garden experiment, we had 4 treatments resulting in 8 treatment combinations replicated 5 times (n = 40) in a randomized block design. Plants were grown in the common garden. In the P5 experiment, the 8 treatment combinations were replicated 2 times (n=16). Plants were grown in the greenhouse.

Harvest and Size Measurements

The garden experiment was carried out from May 2009 to September 2009 in the common garden of the Institute of Plant Biology of the University of Zürich, Switzerland. For harvesting biomass, all leaf material from rosettes and stolons that exceeded a length of 5 cm was cut with scissors. Stolons growing out of the limits of the boxes were cut off and collected separately. Stolon number was counted and the length of the longest stolon of each plant was measured as a proxy for maximal vegetative spread. For measuring vegetative spread, stolons were not stretched out to be measured, but remained in their positions in the boxes.

The P5 experiment was carried out in the greenhouse of the Institute of Plant Biology of the University of Zürich in Zürich, Switzerland in winter 2010/2011. At the end of the experiment, entire plants without roots were harvested and their biomass was measured. Stolons were counted and stretched out their length. To measure biomass, harvested biomass was oven-dried for 48 hours at 80 °C and weighed to the nearest 0.1 g.

Ploidy Analysis

The ploidy level of experimental plants was controlled by ploidy analysis following the two-step method described by Dolezel and colleagues (2007) with minor modifications. Leaf material from stolons was chopped with a razor blade in 5 cm diameter petri dishes in 500 μL of 0.1 M citric acid (Fluka, Buchs, Switzerland), 0.5% Triton X-100 (Sigma-Aldrich, Steinheim, Germany). The solution was filtered through 30 μm filters (CellTricsTM, Partec, Görlitz, Germany) into 1.5 ml Eppendorf tubes (Sarstedt, Numbrecht, Germany). Nuclei were collected by centrifugation at 200 g for 5 min at room temperature (Centrifuge 5415D, Eppendorf, Schönebuch, Switzerland). The supernatant was removed and nuclei were resuspended in 40 μL 0.1 M citric acid, 0.5% Triton X-100. One hundred and sixty μL of staining solution (0.4 M Na_2HPO_4 (Merck, Darmstadt, Germany), 5.5 μg/mL DAPI (4`,6-diamidino-2-phenylindole, Invitrogen, Eugene, Oregon, USA), 0.2 μL/mL 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany)) were added 2 min prior to analysis by the flow cytometer robotics (Quanta SC MPL, Beckman-Coulter, Nyon, Switzerland).

Statistical Analyses

Separate statistical analyses were carried out for each measured trait and the two experiments. We used linear mixed models with “Box” and “Replicate” as random factors and summarized results in Analysis of Variance (ANOVA) tables. The final model was selected based on backward elimination of non-significant terms ($\alpha > 0.05$), with keeping

non-significant terms if they were part of a significant interaction. Biomass had to be ln-transformed to normalize residuals. Stolon number and stolon length could be analyzed without transformation.

First, an overall analysis of the complete dataset was performed. This analysis also tested for differences in the response of apomicts and sexuals to within- and/or between-species competition. Such differences are indicated by a significant interaction between the treatment “Reproduction” and the treatments “Between-Species Competition” and/or “Within-Species Competition” (Fig 1A, 1B).

Second, to test for differences between apomicts and sexuals in particular treatment level combinations the dataset was split. This separation was necessary to correctly interpret effects when significant interactions between factors occurred. The splitting and the analysis were done in two steps. First, the between-species competition treatment was divided into its two levels (Fig 1B i,ii & 1B iii,iv), which were analyzed separately. This step tested for differences in the response of apomicts and sexuals to within-species competition. Second, the two data-subsets were divided into the two levels of the within-species competition treatment. This resulted in four different data-subsets of the 4 treatment level combinations. These data-subsets were separately used to test for differences between apomicts and sexuals in each particular treatment level combination.

Third, testing for differences between the different lineages used in the experiments, the analysis part 1 described above was repeated with the treatment “Lineage” instead of “Reproduction”. Of the three sexual lineages in this experiment, “J2” seemed to perform as well as the aP5 lineages together. Following this analysis, a data-subset was selected which contained only the apomictic aP5 plants from New Zealand and plants of the lineage “J2” (sexual tetraploid). This data-subset was then analyzed again as described above. In this third part of the analysis we estimated if the observed effects were genotype-dependent or not.

Analyses were performed in R (R Developmental Core Team, 2010) using the nlme package (Pinheiro et al., 2009) and the ggplot2 package (Wickham, 2009).

Results

No Competition

When competition was absent, no differences were found for biomass and length of the longest stolon between apomictic and sexual plants in both experiments. The number of stolons, however, was significantly different in the garden experiment with apomicts producing 1.32 times the number of stolons of sexuals ($P = 0.028$, Table 2). This was not found in the P5 experiment, indicating that the higher number of stolons of apomicts was influenced by the ploidy level, but not by the reproductive mode. These results indicate that, except for the number of stolons, there is no basic difference between the reproductive types for the measured traits.

Between-Species Competition

In both experiments the presence of grass as a competitor affected biomass, stolon count and stolon length negatively for both apomicts and sexuals (Table 1, rows “Between-Species”, Fig. 2). We also found that apomicts always performed better than sexuals in both experiments (Table 2, Fig. 2).

Both experiments revealed different responses to between-species competition of apomicts and sexuals for stolon count and longest stolon (Fig. 2, Table 1, rows “Between-Species:Reproduction”). Stolon count and stolon length in apomicts decreased more in the presence of grass than it did in the sexuals (Fig. 2 B, C), indicating that sexuals were better competitors in terms of vegetative propagation and spread.

However, in the analysis of particular treatment combinations we found no differences between apomicts and sexuals in between-species competition, probably because the competition pressure was not big enough to result in a significant difference between apomicts and sexuals.

Within-Species Competition

A different reaction of apomicts and sexuals was found in the P5 experiment for biomass (Fig. 2 A ii, Table 1 b, row “Within-Species:Reproduction”) only. In this case, apomicts were affected less than sexuals by the presence of plants of the other reproductive type. In fact, apomicts increased their biomass under within-species competition, while sexuals reacted negatively. This indicates that apomicts were better within-species competitors in terms of growth.

In the garden experiment, apomicts were 2.08 times heavier than sexuals ($P < 0.001$, Table 2 a). This was similar in the P5 experiment, where apomicts were 3.14 times heavier than sexuals, indicating the superiority of apomicts in competition with sexuals ($P = 0.018$, Table 2 b, Fig. 2 A ii). In the case of the P5 experiment, we attribute this difference to the positive reaction of apomicts to within-species competition (see above).

In the garden experiment, apomicts produced 1.33 times the number of stolons of sexuals when they competed with each other ($P < 0.001$). However, this difference was as big as in the no-competition treatment (Table 2 a), indicating that it was not an effect of competition but of the different ploidy level. However, a similar difference was found in the P5 experiment. Here, apomicts produced 2.0 times as many stolons as sexuals ($P = 0.049$, Table 2 b), while no difference was observed in the no-competition treatment. Although the interaction "Within-Species:Reproduction" was not significant in the overall analysis (Table 1 a and b), we attribute the difference to the different reproductive modes, confirming that apomicts are better within-species competitors.

Apomicts had 1.14 times longer stolons than sexuals in the garden experiment ($P = 0.034$, Table 2 a) and 1.89 times longer stolons than sexuals ($P = 0.001$, Table 2 b) in the P5 experiment. We tentatively conclude that the difference is due to the reproductive mode *per se*, and that apomicts are better within-species competitors.

Combined Between- and Within-Species Competition

When apomicts were compared with sexuals while competing with grass and at the same time competing with each other, apomictic plants produced 1.06 times the number of stolons than did sexuals ($P = 0.019$, Table 2 b). This was found in the P5 experiment only. We conclude that apomicts had a fitness advantage in terms of vegetative reproduction in a complex community, despite the fact that sexuals reacted less to between-species competition (significant interaction "Between-Species:Reproduction" in the overall analysis, Table 1 a and b).

Effects of Lineage (Genetic Background)

In the garden experiment, three different tetraploid sexual lineages were compared with several apomictic pentaploid lineages from an invasive population in New Zealand. Analyzing the different lineages instead of the different modes of reproduction showed that one of the three sexual tetraploid lineages (J2, light grey boxes in Fig. 3) performed similarly well as all aP5 lineages from New Zealand together (dark grey boxes in Fig. 3), whereas the other two sexual lineages behaved as described above (Fig. 3). J2 was the better between-species competitor in terms of stolon count when compared with the aP5

lineage from New Zealand (P = 0.02 for interaction term). No other differences between the apomictic New Zealand lineages and the sexual J2 lineage were found. These results suggest that sexual plants are generally better between-species competitors, at least in terms of vegetative reproduction, irrespective of their genotype.

Discussion

We aimed to compare successful apomictic *H. pilosella* plants from New Zealand with sexual *H. pilosella* from Europe in order to determine whether the success of the New Zealand lineages is, at least in part, due to a fitness advantage in growth (biomass) and vegetative propagation (stolon count and maximum stolon length). Sexual *H. pilosella* were better between-species competitors in terms of number of stolons and length of the longest stolon if compared to apomictic *H. pilosella*. This suggests that sexual populations are more stable than apomictic populations in complex, temporally variable communities compared to apomictic plants. On the other hand, apomictic *H. pilosella* had a higher biomass, more stolons and longer stolons than sexual plants in co-occurrence with sexual *H. pilosella*. This result suggests that sexual plants may be displaced by apomictic plants if competing with each other.

Under between-species competition we found no significant differences between apomicts and sexuals even though sexuals were not as negatively affected by the presence of grass as apomicts were. It is possible that the competition pressure in the experiment was not high enough to result in a significant difference when grass was present. It is, however, conceivable that sexuals would compete better in the long term and under stronger between-species competition, since sexuals were more stable under between-species competition (Fig. 2)

When apomicts and sexuals competed with each other, apomicts were found to be superior to sexuals for all three traits, irrespective of their ploidy level. For biomass, this is in concordance with a study in *Taraxacum sp.*, in which the apomictic triploid plants had a higher biomass than sexual diploid plants, if both types competed directly with each other (De Kovel, 2001). We conclude that apomicts have a better ability for vegetative reproduction, which gives them an advantage over sexuals in the invasion process.

If apomicts competed with sexuals and at the same time with grass, apomicts produced more stolons in the P5 experiment only, making the apomicts the better competitors. Surprisingly, the increased number of stolons was not found in the garden experiment, in which the apomicts had a higher ploidy. We would expect to observe this effect in both experiments, or even more pronounced in the garden experiment, if the effect depended on the ploidy level. One possible explanation for this discrepancy is that the sP5 lineages were not efficient competitors, since no competitive selection had occurred in the history of these lineages, in contrast to isolates from wild populations. However, the sP5 lineages were directly derived from such wild populations. Another

possible explanation might be that beneficial allele combinations of the parents have been disrupted in the sP5 lineages (hidden cost of sex). A third possibility is a genetic association of enhanced vegetative reproduction with apomixis. This would need testing in further separate experiments. We found a ploidy effect only on the number of stolons in the no-competition treatment.

We used heterogenous offspring, i.e. several different lineages, from only one apomictic population from New Zealand in our experiment. It is possible that plants from other populations might behave differently and would not exhibit the same superior vegetative properties that we observed. In fact, we had one sexual lineage in our experiment that grew as vigorous as the apomictic ones. This suggests that, independent of the reproductive mode, the genetic background is important for the competitive behavior of *H. pilosella*.

The observed differences in between-species competition, namely that apomicts produced more stolons and longer stolons, but reacted more negatively to between-species competition than sexuals, supports the hypothesis that apomictic plants are pioneers that are replaced by sexual plants the older and the more complex the community environment becomes (geographic parthenogenesis, Tomlinson, 1966; Mráz et al., 2008). It also implies that sexual populations might have an advantage in variable and rapidly changing environments. Such a distribution pattern can be found across Europe for *H. pilosella* with apomictic cytotypes (pentaploids) found in Northern Europe and sexual cytotypes (tetraploids) found in central Europe (Mráz et al., 2008), which appears to be in concordance with the ice-cover of the latest glaciation in Europe (Mráz et al., 2008). Besides different competitiveness, the cytogeography of *H. pilosella* in Europe could be explained either by the general-purpose genotype hypothesis or the destabilizing hybridization hypothesis (Lynch, 1984). Another possibility is that the niches occupied by apomicts did not change since the glaciers retreated (frozen-niche variation hypothesis, Vrijenhoek, 1979; Vrijenhoek, 1984). In New Zealand, repeated hybridization of *H. pilosella* with *H. praealtum* resulted in genetic variation, enabling the invasion of different niches. Chapman and colleagues (2000) have shown that the genetic diversity within apomictic populations in New Zealand is low, but also that there is no correlation between geographical and genetic distance. They report that genetic variation between populations is higher than within populations, but the variation is low also between populations.

Our findings that plants of the sexual sP5 lineages performed less well than its sexual parental line J2 under within-species competition support the destabilizing hybridization theory, since the beneficial combination of alleles in the sexual parent (J2)

was lost upon fertilization/hybridization. Our findings suggest furthermore that repeated hybridizations and a particular selection regime have led to the creation of new, successfully spreading genotypes in New Zealand. Better vegetative reproduction and better vegetative spread could be attributes of a general-purpose genotype, which could then be fixed by apomixis. Thus, our's and Chapman's findings can be interpreted as supporting the general-purpose-genotype hypothesis.

Summary

In this study, we showed that apomixis and – independently of the mode of reproduction – the ploidy level can affect the fitness of *H. pilosella*. Up to now we cannot distinguish whether the observed advantage of the New Zealand apomicts is solely due to the mode of reproduction, or if and to which extent it is due to the different genetic background of the plants from New Zealand. Clearly, our results suggest that the success of the invasive New Zealand lineages is due to a fitness advantage in growth and vegetative propagation compared to the sexual lineages as they are mostly found in Europe. Interestingly, sexual plants were found to be better between-species competitors, indicating that apomictic *H. pilosella* are favored in pioneer habitats but might be displaced with ongoing succession by sexual plants. More apomictic and sexual lineages will have to be analyzed to fully disentangle the effect of the genetic background from the effect of the mode of reproduction.

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Tables

Table 1: Treatment effects on biomass, stolon count and longest stolon from overall ANOVA of final linear-mixed-effects models in two competition experiments. No interaction of all three factors was found. A) Garden experiment; apomictic and sexual plants differ in ploidy, B) P5 experiment; apomictic and sexual plants have equal ploidy. * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$

A)

	ln(Biomass)				Stolon Count				Longest Stolon			
	num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value
(Intercept)	1	115	1689.49	<0.001***	1	198	484.81	<0.001***	1	134	1463.96	<0.001***
Between-Species	1	26	40.39	<0.001***	1	32	70.75	<0.001***	1	31	46.36	<0.001***
Within-Species	1	26	0.00	0.968	1	32	1.03	0.317	1	31	2.11	0.157
Reproduction	1	115	49.29	<0.001***	1	198	14.32	<0.001***	1	134	3.58	0.061
Between-Species:Reproduction	1	115	0.15	0.695	1	198	7.97	0.005**	1	134	5.12	0.025*
Within-Species:Reproduction	1	26	2.35	0.138	1	32	0.03	0.861	1	31	0.38	0.540

B)

	ln(Biomass)				Stolon Count				Longest Stolon			
	num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value
(Intercept)	1	43	2555.39	<0.001***	1	27	162.74	<0.001***	1	27	366.14	<0.001***
Between-Species	1	11	9.53	0.010*	1	11	11.78	0.006**	1	11	11.17	0.007**
Within-Species	1	11	0.01	0.925	1	11	1.61	0.231	1	11	2.28	0.159
Reproduction	1	43	5.01	0.030*	1	27	8.32	0.008**	1	27	13.20	0.001**
Between-Species:Reproduction	1	43	0.09	0.763	1	27	5.77	0.024*	1	27	5.29	0.029*
Within-Species:Reproduction	1	11	5.00	0.047*	1	11	0.05	0.834	1	11	2.32	0.156

Table 2: Differences in means and coefficients of variance (CV) for biomass, stolon count, and stolon length among apomicts and sexual plants due to particular treatments (without competition, within-species competition, between- and within-species competiton). A) Garden experiment; apomictic and sexual plants differ in ploidy, B) P5 experiment; apomictic and sexual plants have equal ploidy. * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$

A)

Experiment	Trait	Competition Treatment	apomict (CV)	sexual (CV)	p-value
Garden	biomass [mg]	within-species	851 (0.44)	349 (1.05)	< 0.001***
	stolon count	none	7.5 (0.33)	5.6 (0.29)	0.028*
		within-species	7.6 (0.29)	5.7 (0.32)	< 0.001***
	longest stolon [mm]	within-species	477 (0.22)	415 (0.27)	0.034*

B)

Experiment	Trait	Competition Treatment	apomict (CV)	sexual (CV)	p-value
P5	biomass [mg]	within-species	3105 (0.59)	989 (0.59)	0.018*
	stolon count	within-species	8.6 (0.41)	4.4 (0.34)	0.049*
		between- & within-species	4.6 (0.64)	4.3 (0.37)	0.019*
	longest stolon [mm]	within-species	343 (0.22)	182 (0.27)	0.001**

Figure Legends

Figure 1: (A) Design of experiments testing differences among apomictic and sexual lineages of *Hieracium pilosella*, depicting the 3 levels of the experiment. (B) Arrangement of apomictic and sexual *H. pilosella* and the grass *Bromus erectus* in four treatment combinations: (i) no competition, (ii) within-species competition, (iii) between-species competition, and (iv) within- and between-species competition. Left: the experiment with plants of different ploidy (Garden experiment), which was performed in the common garden with 5 replicates in a randomized block design. Right: the experiment using plants of equal ploidy (P5 experiment), which was performed in the greenhouse in duplicate in a randomized block design. Yellow circle – sexual plant; red circle – apomictic plant; green circle – grass

Figure 2: Box and whisker plots of (A) ln(biomass), (B) stolon count, and (C) maximum stolon length of two experiments showing a different reaction (significant interaction) of apomictic and sexual *H. pilosella* to competition. No significant differences were found for ln(biomass) in the Garden experiment. Grey: apomictic plants from New Zealand; (i) sP4 *vs* aP5 plants (“Garden” experiment), (ii) sP5 *vs* aP5 plants (“P5” experiment).

Figure 3: Box and whisker plots of A) ln(biomass), (B) stolon count, and (C) maximum stolon length testing the effects of within- and between-species competition of several apomictic lineages and three sexual lineages (J2, J3, M1) of *H. pilosella*. The clones of each reproductive mode differ in their ploidy level. In all panels 0 refers to absences and 1 refers to presence of competition. 0 and 1 refer to between-species competition at the top of the panels and to within-species competition at the bottom of the panels. Light grey boxes – the sexual lineage J2; middle grey boxes – sexual lineages J2 and M1; dark grey boxes – the apomictic aP5 lineages

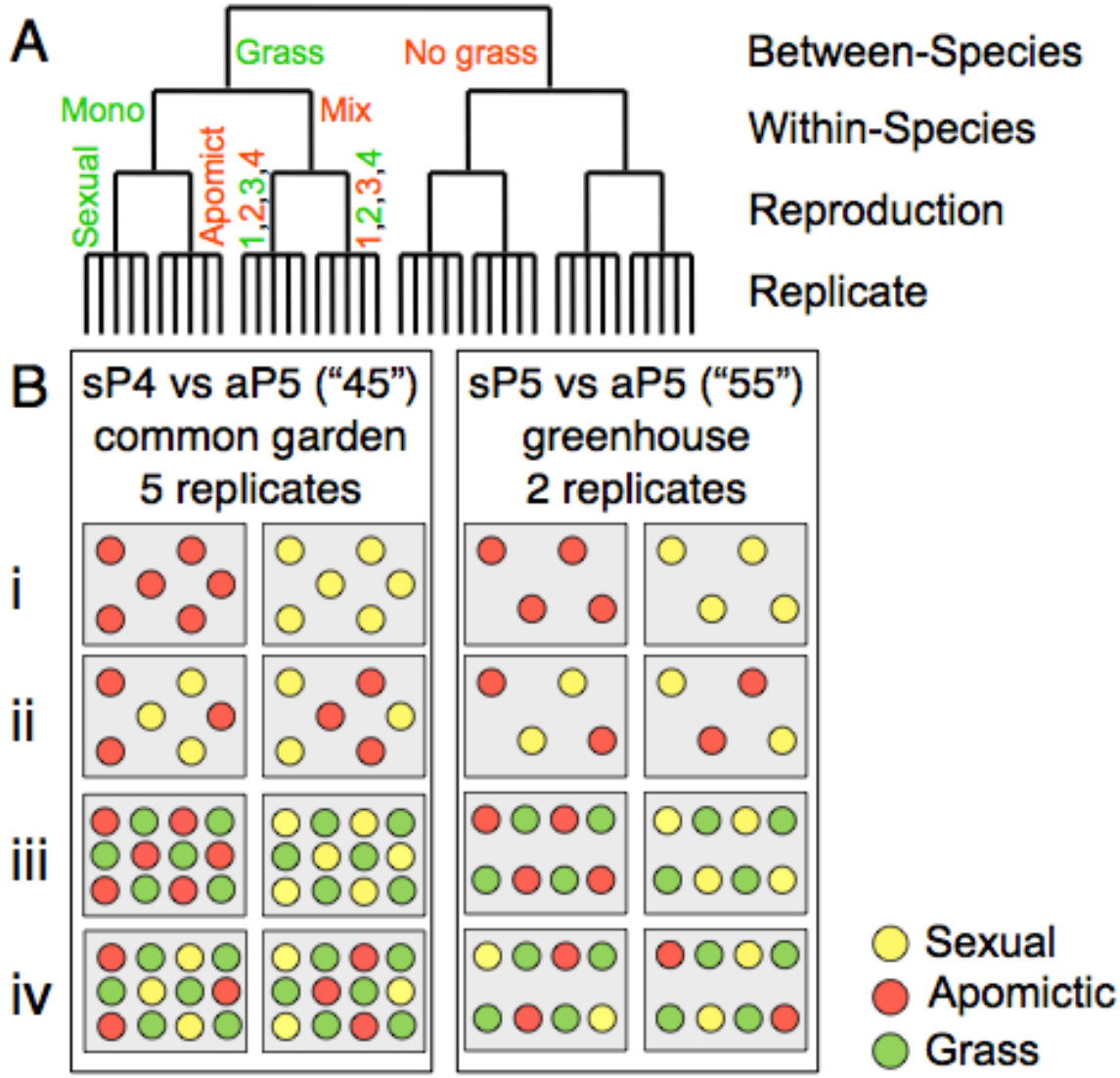
A
Grass
No grass
Mono
Mix
Sexual
Apomict
1,2,3,4
1,2,3,4
Between-Species
Within-Species
Reproduction
Replicate
B
sP4 vs aP5 ("45")
common garden
5 replicates
sP5 vs aP5 ("55")
greenhouse
2 replicates
i
ii
iii
iv
Sexual
Apomictic
Grass

Figure 1

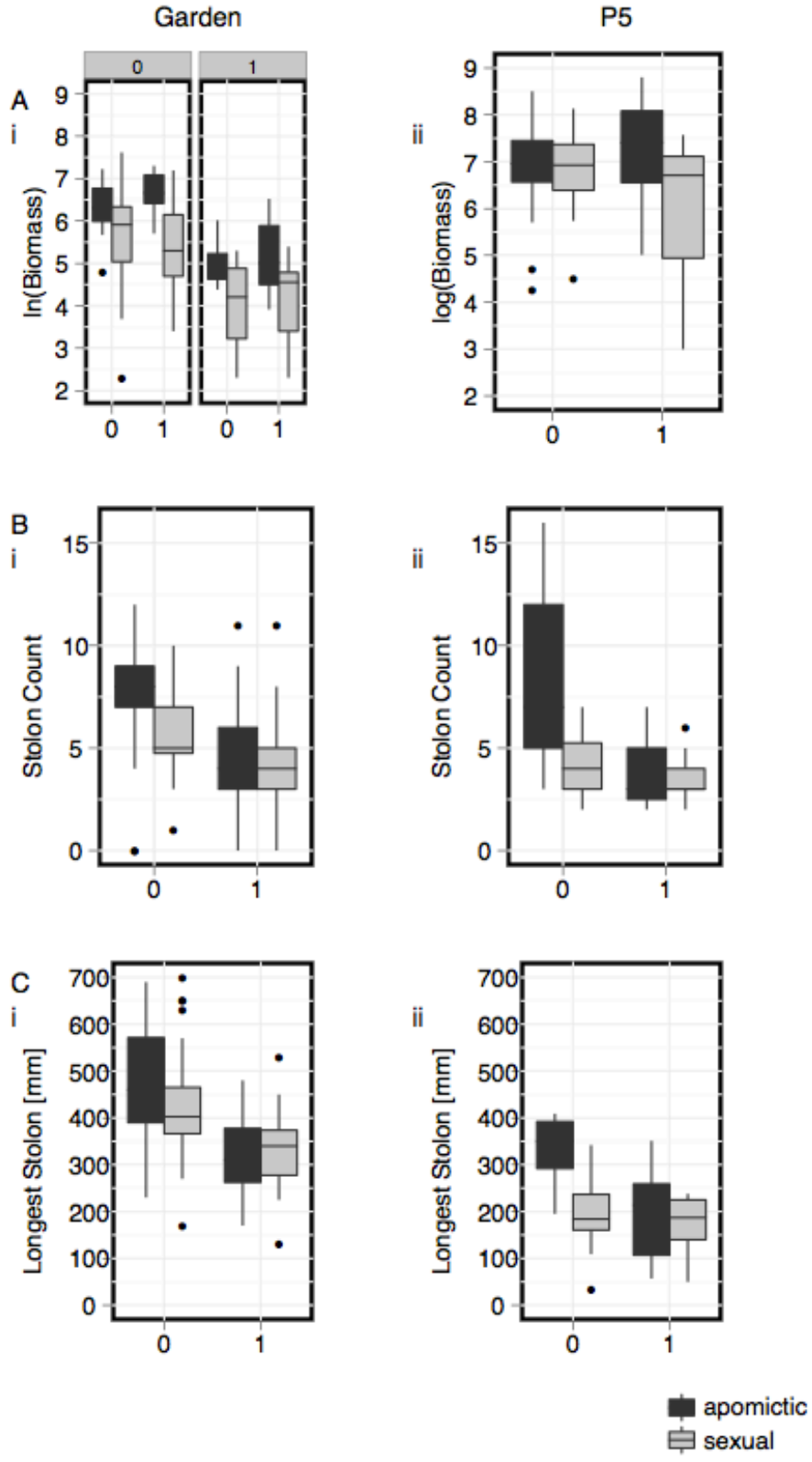
Garden
P5
0
1
A
i
ii
ln(Biomass)
log(Biomass)
B
Stolon Count
C
Longest Stolon [mm]
apomictic
sexual

Figure 2

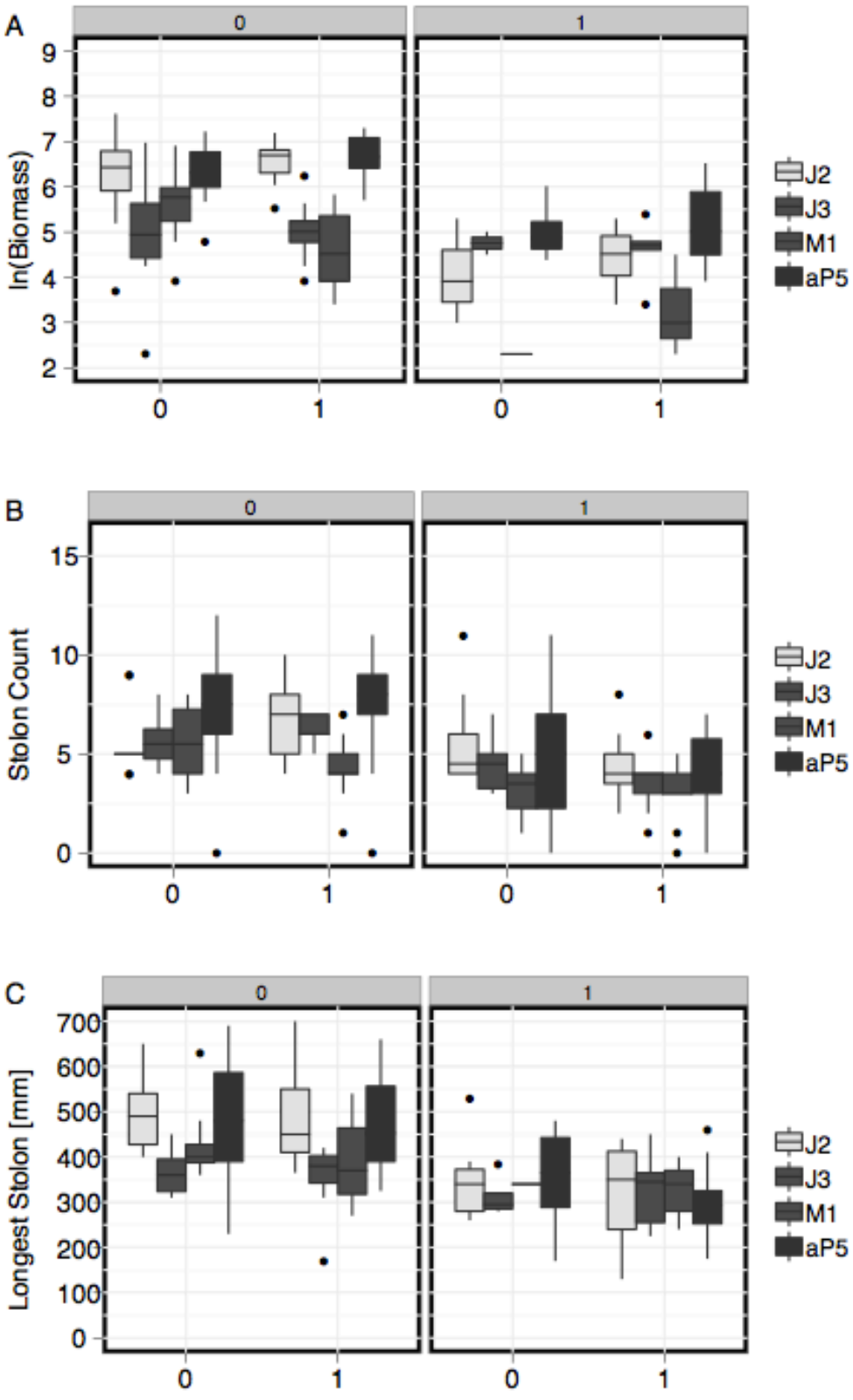
A
0
1
ln(Biomass)
9
8
7
6
5
4
3
2
0
1
0
1
J2
J3
M1
aP5
B
0
1
Stolon Count
15
10
5
0
0
1
0
1
J2
J3
M1
aP5
C
0
1
Longest Stolon [mm]
700
600
500
400
300
200
100
0
0
1
0
1
J2
J3
M1
aP5

Figure 3

Behavior of Apomictic and Sexual Siblings in Different Competition Settings

Behavior of Apomictic and Sexual Siblings in Different Competition Settings

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Abstract

Apomixis, the asexual reproduction through seed, is reported in over 40 plant families and leads to avoidance of the hidden cost of sex. Apomictic plants have an advantage in sparse population densities and in colonizing new areas, but might have a disadvantage in changing environments, since they reproduce with a fixed genotype.

In this study we compared apomictic and sexual siblings of the *Hieracium pilosella* L. in different competition settings. We measured 12 different fitness related traits and analyzed if observed differences are due to the mode of reproduction or the genetic background (unrelated genotype).

Apomicts and sexuals performed differently for all traits in different competition settings except for viability. In all cases except for individual seed mass, the unrelated genotype was of importance for the effect. Furthermore we present evidence for better resistance to competition of genetically more diverse populations and showed that the selfing syndrome (smaller floral display in selfing species than in outcrossing sister species) occurs in apomicts.

Our results suggest that selection for fitness traits in different competition settings happens on the whole genome and that apomixis provides an additional resource for variation.

Introduction

Apomixis, the asexual reproduction through seeds, occurs in over 400 plant species of 40 different families (Asker and Jerling 1992). Apomicts produce pollen and can outcross. Apomixis is a facultative trait (Asker and Jerling 1992), meaning that is does not lead to avoidance of the cost of sex (Maynard-Smith 1971), since the male gametophyte is still produced. However, sexual reproduction results in offspring which are new genotypes. Not all offspring will survive, since not all genotypes are adequately adapted to the current environment. This can be referred to as the hidden cost of sex, which is avoided in apomicts. Apomixis results in maternal clonal offspring. It can be assumed that these are genotypes, which are well adapted to the current environment , since they survive and reproduce. Apomixis provides reproductive assurance (Darwin 1862, Baker 1955, Tomlinson 1966), which in turn leads to an advantage in colonization and dispersal, as the apomicts do not need to find a mate to reproduce (Baker 1955, Tomlinson 1966).

A good example of an apomictic species is *Hieracium pilosella* L., an autonomous aposporous apomict, which has successfully invaded New Zealand (Scott et al. 1990, Connor 1992, Chapman et al. 2000, Houliston and Chapman 2004) and currently is invading Patagonia (Krahulcová and Krahulec 2011). In both cases, the prevalent cytotype is apomictic pentaploid (aP5, apomictic Pilosella 5-ploid). In theory, according to Baker's law, a single individual is sufficient to found a new population (Baker 1955). However, stochastic demographic effects make the establishment of a new population from a single propagule highly unlikely. In fact, in New Zealand, *H. pilosella* was introduced several times and hybridized with the closely related *H. praealtum* (Morgan-Richards et al. 2004, Trewick et al. 2004), creating new genotypes, which were selected for spread.

In a recent study it was shown that these aP5 cytotypes were superior competitors in comparison to their sexual European ancestors (Sailer et al., submitted, Morgan-Richards et al. 2004, Trewick et al. 2004). However, the influence of the mode of reproduction and the influence of the genetic background (we will refer to it as "unrelated genotype" in this paper) was not resolved. To address this, we created apomictic and sexual siblings by crossing sexual hexaploid plants from Europe with apomictic hexaploid plants from New Zealand and repeated the competition experiment with their vegetative progeny. We found that the previously observed effects of successfully competing apomicts were not solely due to the mode of reproduction, but that selection of successful competition in apomicts occurred on the overall genotype in New Zealand. Using apomictic and sexual siblings from different families enabled us to estimate 12 fitness parameters (Table 1). We found a

general difference between apomicts and sexuals in 2 of the 12 parameters tested. However the unrelated genotype was of general influence as well. Furthermore we provide evidence for reproductive assurance of apomicts and show that the selfing syndrome (selfing species have a smaller floral display than their out-crossing sister species (Sicard and Lenhard 2011)) occurs in apomicts.

Materials and Methods

Plant Material

Hieracium pilosella L. is a self-incompatible, perennial, monocarpic herbaceous species including sexual and apomictic lineages, which can occur at different ploidy levels. Plants can reproduce vegetatively via aboveground stolons. Apomictic lineages are of the autonomous apospory type, and apomicts can outcross via pollen.

Three sexual hexaploid lines (sP6, sexual Pilosella 6-ploid) were isolated from three populations from the Morteratsch proglacial area, Upper Engadin, Switzerland (MoK: 791849, 145561; MoG20 & MoG23: 792087, 148071; Swiss Grid). Two apomictic hexaploid lines (aP6, apomictic Pilosella 6-ploid) were isolated from two populations in New Zealand (line LaP1, Lake Pukaki, latitude: -44.15848, longitude: 170.22020 and line MwR1, Molesworth Road, latitude: -42.00933, longitude: 172.95406). Line LaP1 had low apomictic fertility (low), while line MwR1 had high apomictic fertility (high). The two apomictic lines were used as pollen donor and each was crossed with the three sexual lines, resulting in 6 different families. The F1 progeny of these crosses were grown in the greenhouse and tested for apomixis by decapitation (Koltunow et al. 1995). This way apomictic and sexual siblings were generated, together with their half siblings (different paternal genotype) resulting in 4 different genotypes: i) apomictic high (Ah), ii) apomictic low (Al), iii) sexual high (Sh), and iv) sexual low (Sl). Plants were propagated vegetatively to generate 8 (8 treatment combinations are 1 replicate, see experimental design) vegetative clones per line.

Propagation of Lines and Soil

After surface sterilization, F1 seeds were germinated in petri dishes on half strength MS-medium (Murashige and Skoog 1962) (containing MS salts (Carolina, Burlington, North Carolina), Sucrose (Applichem, Darmstadt, Germany) and Phytoagar (Gibco BRL, Paisley, Scotland)) in a Percival Scientific climatic cabinet (CU-36L6/D, CLF Plant Climatics GmbH, Wertingen, Germany) at 22°C/18°C (day/night) 14h light and 10h dark cycle. When seedlings had produced two to three true leaves, they were transferred to a nutrient-poor soil (“Dachgartenerde extensiv”, Ricoter Erdaufbereitungs AG, Frauenfeld, Switzerland), to mimic the field condition. Seedlings were grown in the greenhouse at 20°C/16°C (day/night) for three days under a humidor.

Stolons of apomictic and sexual plants were cut and put onto soil (“Aussaaterde”, Gebr. Patzer GmbH & CoKG, Sinntal-Altengronau, Germany) under a humidor until the

plants had produced roots. These plants were then transplanted into plastic boxes (Georg Utz AG, Bremgarten, Switzerland) of 40 x 30 x 30 cm (L x W x D), which were filled with a nutrient poor soil ("Dachgartenerde extensiv", Ricoter Erdaufbereitungs AG, Frauenfeld, Switzerland), to mimic the field condition. The bottom of the boxes had holes and was covered with a 2 cm thick drainage mat to prevent root rotting in standing water.

Experimental Design

Apomictic and sexual sibling pairs were chosen randomly from each family. Sibling pairs derived from a cross with the high apomixis father (Ah-Sh) were randomly combined with sibling pairs from a cross with the low apomixis father (Al-Sl). This combination of genotypes was defined for each replicate. High and low plants were always grown together (2 levels of – to the mode of reproduction – unrelated genotype). Apomictic and sexual siblings (2 levels of reproduction) were grown either alone or together with the other reproductive type (within-species competition), and with or without the grass *Bromus erectus* Huds. (between-species competition). *B. erectus* (from the Swiss Jura Mountains; Otto Hauenstein Samen, Rafz, Switzerland) served as a novel between-species competitor. This resulted in 4 different treatment combinations: 1) no competition, 2) between-species competition, 3) within-species competition, and 4) both between- and within-species competition.

Plants were grown in plastic boxes (see above). The boxes were covered with mosquito net (Windhager AG, Baar, Switzerland) cages to prevent pollination between experimental units in the common garden. Boxes with a cage were considered as experimental units. In each box, positions of 4 *H. pilosella* plants were fixed in a grid of 8 positions. Individuals of the defined genotype combinations were randomly assigned to these positions. For between-species competition 4 *H. pilosella* plants were grown alternating with 4 plants of *B. erectus*. For within-species competition, sexual and apomictic plants of the 4 genotypes (Ah, Al, Sh, Sl) of the defined genotype combinations were planted alternately in a ratio of 1:1. To control for position effects, the order was switched in every second experimental unit. The 4 treatments resulted in 8 treatment combinations, which were replicated 5 times in a fully randomized design.

Crossing, Measurements and Harvest

Plants were grown in the common garden of the Institute of Plant Biology of the University of Zürich, Switzerland, from April 2012 to September 2012. At the day of opening of the capitulum its diameter was measured to a precision of 1 mm. All capitula of

all individuals of 1 experimental unit, which were open at the same time, were crossed with each other by rubbing 2 capitula together. The crossings were repeated every day until closing of capitula. At the day of seed set, seeds were harvested and stored at 4°C at 30% humidity until used. The length of the stem was measured to a precision of 1 mm. By the end of August most plants had set seed and were harvested. At harvest, the diameter of living rosettes was recorded to a precision of 1 mm. The number of green leaves of the rosette was recorded. Stolons were stretched out and measured to a precision of 1 mm. To harvest above ground biomass, stolons were cut off and collected separately from plants. To measure biomass, harvested plant material was oven-dried for 48 h at 80°C and weighed to a precision of 0.1 mg.

Harvested seeds and empty seed shells were counted and sorted and separately weighed to a precision of 0.1 mg.

To determine germination rates, up to 20 seeds from 1 randomly chosen capitulum per individual, were surface sterilized and germinated in petri dishes on half strength MS-medium (Murashige and Skoog 1962) (containing MS salts (Carolina, Burlington, North Carolina), Sucrose (Applichem, Darmstadt, Germany) and Phytoagar (Gibco BRL, Paisley, Scotland)) in a Percival Scientific climatic cabinet (CU-36L6/D, CLF Plant Climatics GmbH, Wertingen, Germany) at 22°C/18°C (day/night) 14h light and 10h dark cycle after 72h stratification at 4°C. Germination was counted every day for 7 days.

Computed Fitness Parameters

Viability is the number of flowering plants divided by the number of seedlings planted. The number of ovules was calculated by adding number of seeds and number of empty seed shells (due to abortion/not fertilized). Fertility is the number of mature seeds divided by the number of ovules. Germination is the number of germinated seeds divided by the number of seeds plated. Darwinian fitness is the number of plants which successfully reproduced, divided by the number of ovules of the mother plant. Darwinian fitness was estimated by multiplying viability, fertility and germination rate.

Statistical analysis

A separate analysis was carried out for each of the measured and computed variables. We used analysis of deviance (ANODEV) of generalized linear models (glm). For binary count data (e.g. survived – died; viability, fertility) we used the family function “binomial” and the link function “logit”. For stolon count we used the family function “poisson” and the link function “log” (natural logarithm). If the data was over- or underdispersed the family function was changed to “quasibinomial” and “quasipoisson”,

respectively. If the data was regularly dispersed we used the Chi-square test and in case of under- or overdispersion we used the F-test. For the rest of the variables we used the family function “gamma” with either the link function “log” or “identity”. The best transformation was determined using Box-Cox plots.

First a full model was analyzed with consecutive removal of non-significant interactions. If a significant interaction was determined, the dataset was split into the corresponding subsets of different treatments. An analysis of the subsets was performed separately to determine if differences within these subsets (different treatments) occurred.

The data was analyzed using R (R Developmental Core Team 2010) and the MASS package (Venables and Ripley 2010). Graphs were constructed using the ggplot2 package (Wickham 2009) and the grid package (Murrell 2005).

Results

To estimate competitive abilities of apomictic and sexual siblings, we measured 12 reproduction related phenotypes in 4 categories (growth, vegetative propagation, spread, propagation via seed; table 1).

Table 1. Analyzed Phenotypes of Different Categories

Growth	Vegetative Propagation	Spread	Propagation via Seed
Biomass	Number of Stolons	Stolon length	Fertility
		Maximum stolon length	Number of Ovules
		Stem length	Single Seed Mass
			Diameter Capitulum
			Fitness
			Viability
			Germination rate

Apomicts and sexuals reacted differently to competition treatments in 6 of the 12 phenotypes (biomass, stolon length, maximum stolon length, stem length, fertility, single seed mass), and showed a general difference in 2 (number of ovules and diameter of capitulum).

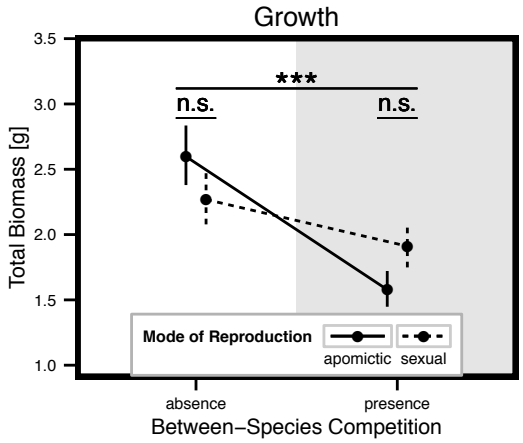
The unrelated genotype showed different reaction to competition treatments in 5 of 12 phenotypes (number of stolons, stolon length, maximum stolon length, fertility, germination rate) and a general difference in 3 (number of ovules, diameter of capitulum, stem length).

The results are presented by category and phenotype, as listed in table 1.

Growth

Biomass is a measure of growth and more vigorous growing plants are expected to be better competitors. Biomass decreased in between-species competition ($F_{1,154} = 15.3$, $p < 0.001$). This is in concordance with previous results (Sailer et al., submitted). Sexuals showed a slightly more stable growth than apomicts (interaction $F_{1,150} = 3.4$, $p = 0.066$), but no difference could be observed within the treatments (Figure 1A). We interpret the more stable growth of sexuals as better resistance of sexuals to between-species competition. Since no differences were found for the unrelated genotype we conclude that more stable growth is linked to the mode of reproduction (Figure 1B).

A



B

	Df	Residual Df	F	Pr(>F)
NULL	NA	155	NA	NA
Between-Species Competition	1	154	15.310	0.0001
Within-Species Competition	1	153	1.637	0.2027
Mode of Reproduction	1	152	0.162	0.6883
Father	1	151	2.483	0.1172
Between : Reproduction	1	150	3.421	0.0663

Figure 1. Growth – Biomass is reduced by between-species competition

A) Biomass is reduced by between-species competition ($F_{1,154}$ = 15.3, $p < 0.001$). Sexuals are more stable than apomicts (interaction $F_{1,150}$ = 3.4, p = 0.066), but no difference between apomicts and sexuals was detectable within the treatment. **B)** Simplified table of ANODEV of a generalized linear model (glm). For the glm the family function "gamma" and the link function "log" were used. Between-species competition affects biomass. There is a weak interaction for between-species competition and the mode of reproduction, indicating that apomicts and sexuals react different to between-species competition.

Grey background – between-species competition; solid line – apomict; dashed line – sexual; n.s. – not significant; · – $p < 0.1$; * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$

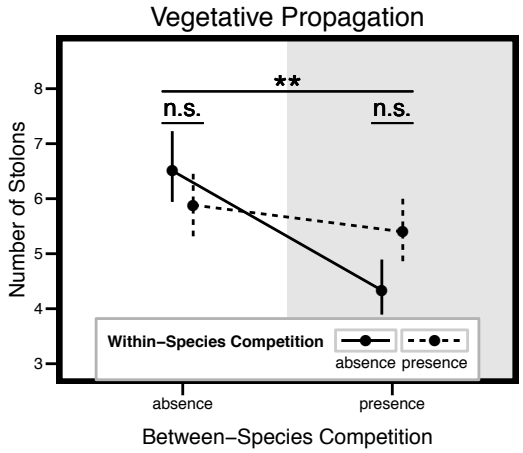
Vegetative Propagation

Vegetative propagation via stolons is the main reproductive pathway in the natural populations (Winkler and Stöcklin 2002).

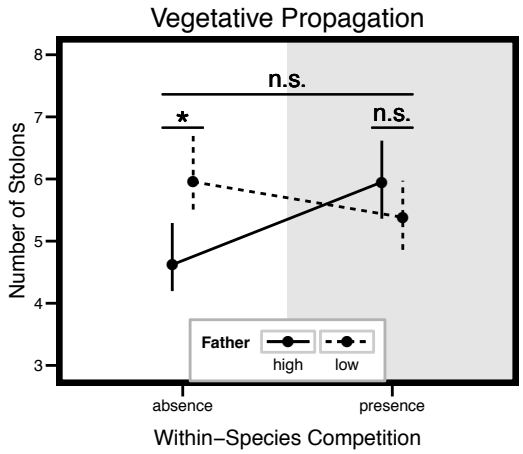
In between-species competition the number of stolons is reduced ($F_{1,147}$ = 7.9, p = 0.006). The magnitude of the reduction is higher for mono-cultures (no within-species competition) than for mix-cultures (within-species competition; interaction $F_{1,143}$ = 3.3, p = 0.07; Figure 2A). Due to the experimental design, the genotypic variance is higher in mix-cultures than in mono-cultures. We attribute the better resistance of mix-cultures to the higher genetic diversity.

Offspring of the high apomixis father had fewer stolons under no competition ($t_{73,1}$ = -2.3, p = 0.026, Figure 2B), compared to offspring of the low apomixis father. Offspring of the high apomixis father slightly increase their stolon count under within-species competition (interaction $F_{1,143}$ = 3.8, p = 0.053), resulting in disappearance of this difference. Since the mode of reproduction did not influence the number of stolons, we conclude that vegetative propagation is unlinked to the mode of reproduction (Figure 2C).

A



B



C

	Df	Resid. Df	F	Pr(>F)
NULL	NA	148	NA	NA
Between-Species Competition	1	147	7.890	0.0057
Within-Species Competition	1	146	0.148	0.7007
Mode of Reproduction	1	145	0.445	0.5060
Father	1	144	0.535	0.4656
Between:Within	1	143	3.325	0.0703
Within:Father	1	142	3.795	0.0534

Figure 2. Vegetative propagation – Between-species competition reduces the number of stolons and mix-cultures are more stable

A) Between-species competition reduces the number of stolons ($F_{1,147}$ = 7.9, p = 0.006). Mixed cultures (apomicts + sexuals) are slightly more stable under between-species competition (interaction $F_{1,143}$ = 3.3, p = 0.070). **B)** Offspring of the high apomixis father increase the number of stolons under within-species competition (interaction $F_{1,143}$ = 3.8, p = 0.053). **C)** Simplified table of ANODEV of a generalized linear model (glm). For the glm the family family function "quasipoisson" was used due to overdispersion of the data, together with the link function "log". Between-species competition affects the number of stolons produced. There is are weak interactions for between-species competition and within-species competition, as well as for within-species competition and the type of father, indicating that the number of stolons depends on the combination of competition treatments and that the unrelated genotypes react differently to within-species competition.
Grey background – between-species competition; solid line – apomict; dashed line – sexual; n.s. – not significant; · – p < 0.1; * – p < 0.05; ** – p < 0.01; *** – p < 0.001

Measures for Spread of Propagules

To measure vegetative spread, the mean and maximum stolon lengths were measured. As a proxy for spread via seeds, the stem length at seed set was measured, based on the assumption that seeds can disperse further from longer stems.

Mean and Maximum Stolon Length

In between-species competition the mean ($F_{1,854}$ = 9.9, p < 0.001; Figure 3A) and the maximum stolon length ($F_{1,147}$ = 22.7, p < 0.001; Figure 3E) are reduced. Mean stolon

length is more stable in apomicts under within-species competition (interaction $F_{1,850} = 5.0$, $p = 0.026$). Moreover, apomicts and sexuals react differently to different competition levels (three way interaction $F_{3,846} = 2.2$, $p = 0.09$; Figure 3B), with sexuals having longer stolons under no competition ($t_{252,1} = 3.5$, $p < 0.001$), within-species competition ($t_{235,1} = 2.9$, $p = 0.004$), as well as under between- and within-species competition ($t_{198,1} = 5.0$, $p < 0.001$; Figure 3B).

Offspring of the high apomixis father increase their mean stolon length under within-species competition (interaction $F_{1,849} = 11.9$, $p < 0.001$). This causes the offspring from the high apomixis father to change from having shorter stolons under no competition ($t_{417,1} = 1.7$, $p = 0.01$) to having longer stolons under within-species competition ($t_{435,1} = 4.3$, $p < 0.001$; Figure 3C).

Sexuals slightly increase their maximum stolon length under within-species competition (interaction $F_{1,143} = 2.9$, $p = 0.090$). This causes sexuals to have a bigger maximum stolon length than apomicts under within-species competition ($t_{72,1} = 2.8$, $p = 0.008$; Figure 3F).

Offspring of the high apomixis father increase their maximum stolon length under within-species competition (interaction $F_{1,142} = 4.3$, $p = .037$), causing a difference between offspring of the high and low apomixis father under within-species competition ($t_{72,1} = 2.5$, $p = 0.016$; Figure 3G).

Since both the mode of reproduction and the unrelated genotype influence stolon length and maximum stolon length, we conclude that vegetative spread is dependent on several loci in the genome.

Stem Length

Stem length, which is a proxy for seed dispersal, is reduced by between-species competition ($F_{1,119} = 9.6$, $p = 0.002$). The reduction is stronger in apomicts than in sexuals (interaction $F_{1,115} = 4.1$, $p = 0.045$; Figure 3D).

Offspring of the high apomixis father have longer stems than offspring of the low apomixis father ($F_{1,116} = 37.3$, $p < 0.001$; Figure 3H).

Both the mode of reproduction and the unrelated genotype influence stem length. We conclude that stem length is dependent on several loci in the genome and that the high apomixis father was selected for longer stems.

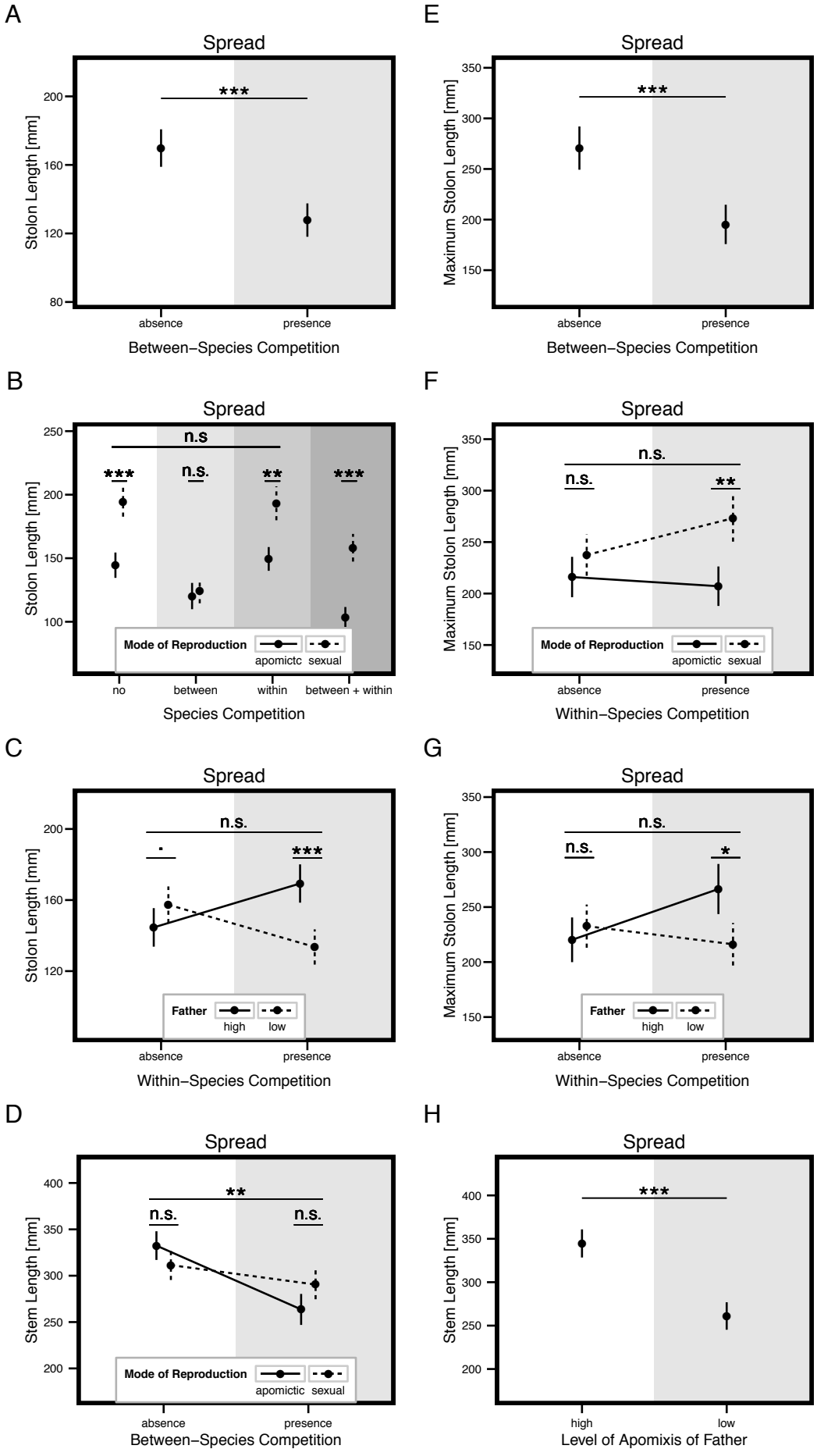

A
Spread
***
Stolon Length [mm]
200
160
120
80
absence
presence
Between−Species Competition
E
Spread
***
Maximum Stolon Length [mm]
350
300
250
200
150
absence
presence
Between−Species Competition
B
Spread
n.s
***
n.s.
**
***
Stolon Length [mm]
250
200
150
100
Mode of Reproduction
apomictc sexual
no
between
within
between + within
Species Competition
F
Spread
n.s.
n.s.
**
Maximum Stolon Length [mm]
350
300
250
200
150
Mode of Reproduction
apomictic sexual
absence
presence
Within−Species Competition
C
Spread
n.s.
.
***
Stolon Length [mm]
200
160
120
Father
high low
absence
presence
Within−Species Competition
G
Spread
n.s.
n.s.
*
Maximum Stolon Length [mm]
350
300
250
200
150
Father
high low
absence
presence
Within−Species Competition
D
Spread
**
n.s.
n.s.
Stem Length [mm]
400
350
300
250
200
Mode of Reproduction
apomictic sexual
absence
presence
Between−Species Competition
H
Spread
***
Stem Length [mm]
400
350
300
250
200
high
low
Level of Apomixis of Father

Figure 3. Spread – Stolon length, maximum stolon length, stem length
A-C) Stolon length **E-F)** Maximum stolon length **D,H)** Stem length **A)** Between-species competition reduced stolon length ($F_{1,854}$ = 39.9, p < 0.001). **B)** Apomicts are more stable in within-species as well as between- and within-species competition (interaction $F_{1,850}$ = 5.0, p = 0.026; $F_{3,846}$ = 2.2, p = 0.090). Sexuals have longer stolons under no competition ($t_{252,1}$ = 3.5, p < 0.001), within-species competition ($t_{235,1}$ = 2.9, p = 0.004), between- and within-species competition ($t_{198,1}$ = 5.0, p < 0.001). **C)** Offspring of the high apomixis father increase their stolon length under within-species competition (interaction $F_{1,849}$ = 11.9, p < 0.001). This results in offspring from the high apomixis father being shorter under no competition ($t_{417,1}$ = 1.7, p = 0.010) to change to being longer under within-species competition ($t_{435,1}$ = 4.3, p < 0.001). **D)** Stem length is reduced under between-species competition ($F_{1,119}$ = 9.6, p = 0.002). Stem length is more stable in sexuals (interaction $F_{1,115}$ = 4.1, p = 0.045). No difference was found between apomicts and sexuals in the different treatments. **E)** Between-species competition negatively influences maximum stolon length ($F_{1,147}$ = 22.7, p < 0.001). **F)** Sexuals slightly increase their maximum stolon length under within-species competition (interaction $F_{1,143}$ = 2.9, p = 0.090) resulting in a difference between apomicts and sexuals under within-species competition ($t_{72,1}$ = 2.8, p = 0.008). **G)** Offspring of the high apomixis father have longer stems than offspring of the low apomixis father ($F_{1,116}$ = 37.3, p < 0.001).
Grey backgrounds – different competition treatments; Grey background in G) – different genotype; n.s. – not significant; · – p < 0.1; * – p < 0.05; ** – p < 0.01; *** – p < 0.001

Propagation via Seed

Apomixis affects seed development (Asker and Jerling 1992, Koltunow and Grossniklaus 2003), which is why we expect to find the majority of differences between apomicts and sexuals for these traits.

Fertility

Fertility is decreased in between-species competition ($F_{1,94}$ = 9.3, p = 0.003). Apomicts show a more stable fertility than sexuals (interaction $F_{1,90}$ = 5.5, p = 0.021), causing apomicts to have a higher fertility than sexuals under between-species competition ($t_{44,1}$ = 2.9, p = 0.005; Figure 4A).

Furthermore, sexuals slightly increase their fertility under within-species competition (interaction $F_{1,88}$ = 3.3, p = 0.071), but no difference could be observed within the different treatments (Figure 4B).

Fertility varies less in apomicts than in sexuals across all competition treatments (Figure 4C).

Offspring of the low apomixis father have a more stable fertility under between-species competition than offspring of the high apomixis father (interaction $F_{1,89}$ = 5.5, p = 0.021). Under no competition, offspring of the high apomixis father have a higher fertility ($t_{48,1}$ = 3.5, p = 0.001; Figure 4D).

The mode of reproduction affects fertility in all treatments, with apomicts being more stable in all treatments. We attribute these results to the reproductive assurance provided by apomixis. The unrelated genotype affects fertility in between-species competition. We interpret these results in the way, that apomixis provides reproductive assurance via stable fertility, which in turn is influenced by other loci in the genome.

Number of Ovules

The number of ovules is a measure for reproductive potential.

Apomicts and offspring of the high apomixis father have more ovules than sexuals and offspring of the low apomixis father, respectively ($F_{1,125} = 15.4$, $p < 0.001$; $F_{1,124} = 5.1$, $p = 0.025$; Figure 4E, F). The four different genotypes decrease gradually in their number of ovules from genotype Ah to Sl ($F_{3,125} = 6.4$, $p < 0.001$; Figure 4G).

We conclude that the different fertility of apomicts and sexuals reflects the different number of ovules. In addition, for the fertility-phenotype several loci in the genome are responsible.

Single Seed Mass

Single seed mass is a proxy for germination success, with heavier seeds having a higher probability of germination ($F_{1,127} = 44.1$, $p < 0.001$).

Single seed mass of apomicts increases under within-species competition (interaction $F_{1,123} = 4.6$, $p = 0.030$). This causes apomicts to have heavier seeds than sexuals in within-species competition ($t_{62,1} = 3.0$, $p = 0.004$; Figure 4H).

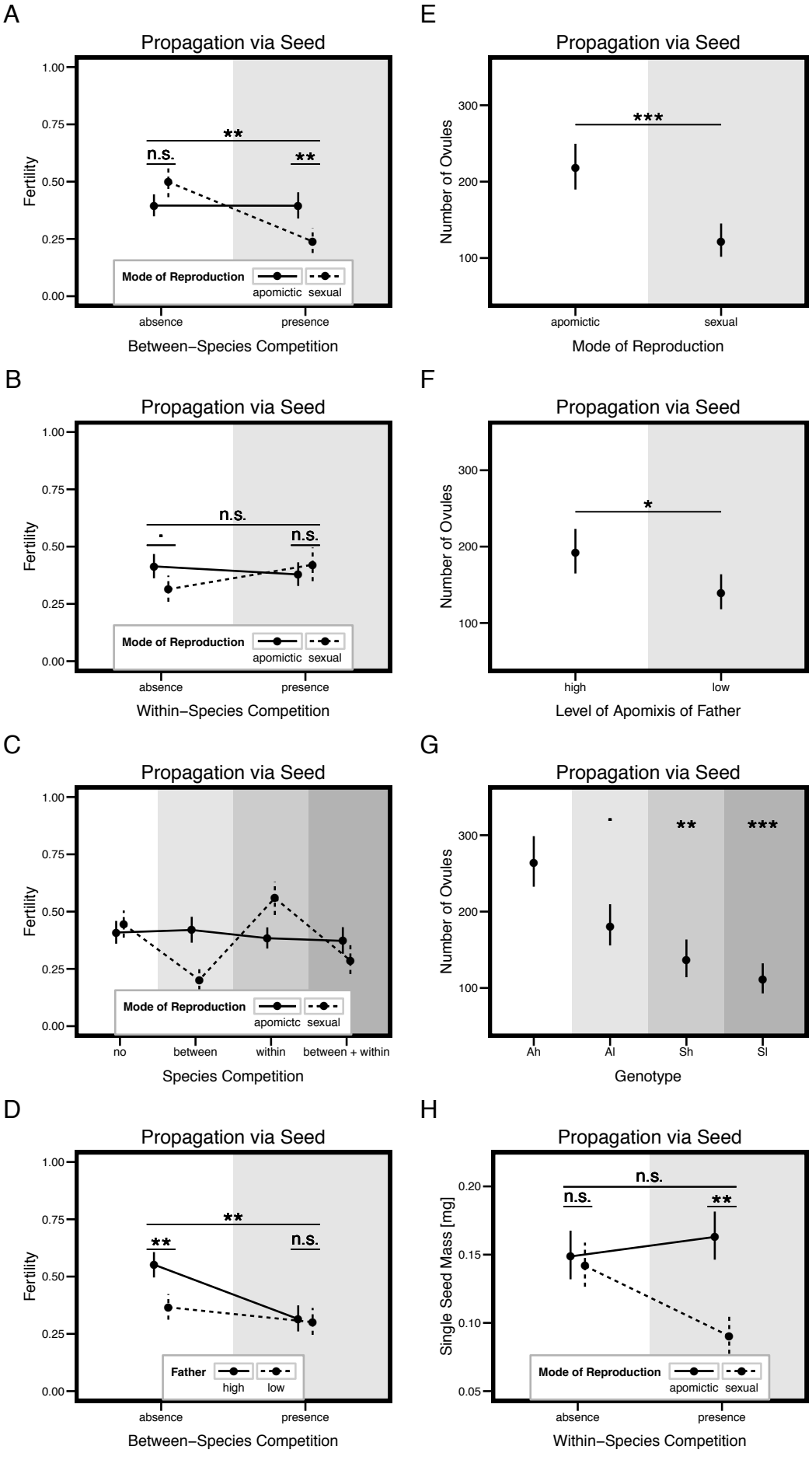
We conclude that offspring size is linked to the mode of reproduction.

Figure 4. Propagation via seeds – Fertility and single seed mass are influenced by competition and apomicts and sexuals react differently

A-D) Fertility (number of seeds / number of ovules) **E-G)** Number of ovules

A, D) Between-species competition reduces fertility ($F_{1,94} = 9.3$, $p = 0.003$). **A)** Between-species competition: Apomicts have a more stable fertility in between-species competition (interaction $F_{1,90} = 5.5$, $p = 0.021$) resulting in apomicts having a higher fertility in between-species competition ($t_{44,1} = 2.94$, $p = 0.005$). **B)** Within-species competition: Fertility of sexuals increases slightly in within-species competition (interaction $F_{1,88} = 3.3$, $p = 0.071$), resulting in disappearance of a slight difference between apomicts and sexuals ($t_{48,1} = 1.7$, $p = 0.009$) under no within-species competition. **C)** Apomicts appear to be more stable across different competition treatments. **D)** Offspring of the low apomixis father are more stable under between-species competition (interaction $F_{1,89} = 5.5$, $p = 0.021$), resulting in disappearance of a difference between low and high apomixis father under no between-species competition ($t_{48,1} = 3.5$, $p = 0.001$). **E)** Apomicts have more ovules than sexuals ($F_{1,125} = 15.4$, $p < 0.001$). **F)** Offspring of the high apomixis father have more ovules than offspring of the low apomixis father ($F_{1,124} = 5.1$, $p = 0.025$). **G)** Gradual difference in the number of ovules of the 4 different genotypes ($F_{3,125} = 6.4$, $p < 0.001$). Genotypes are always compared to the Ah genotype. **H)** Single-seed mass of apomicts increases under within-species competition (interaction $F_{1,123} = 4.6$, $p = 0.030$), resulting in a difference between apomicts and sexuals under within-species competition ($t_{62,1} = 3.0$, $p = 0.004$).

Grey backgrounds – different competition treatments; Grey backgrounds in E-G – different genotypes; n.s. – not significant; · – $p < 0.1$; * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$


A
Propagation via Seed
Fertility
1.00
0.75
0.50
0.25
0.00
**
n.s.
**
Mode of Reproduction
apomictic sexual
absence
presence
Between−Species Competition
E
Propagation via Seed
Number of Ovules
300
200
100
***
apomictic
sexual
Mode of Reproduction
B
Propagation via Seed
Fertility
n.s.
n.s.
Mode of Reproduction
apomictic sexual
absence
presence
Within−Species Competition
F
Propagation via Seed
Number of Ovules
*
high
low
Level of Apomixis of Father
C
Propagation via Seed
Fertility
Mode of Reproduction
apomictc sexual
no
between
within
between + within
Species Competition
G
Propagation via Seed
Number of Ovules
**
***
Ah
Al
Sh
Sl
Genotype
D
Propagation via Seed
Fertility
**
**
n.s.
Father
high low
absence
presence
Between−Species Competition
H
Propagation via Seed
Single Seed Mass [mg]
0.20
0.15
0.10
0.05
n.s.
n.s.
**
Mode of Reproduction
apomictic sexual
absence
presence
Within−Species Competition

Diameter of Capitulum – Selfing Syndrome

The diameter of the capitulum is a measure for the size of the floral display, which attracts pollinators.

Apomicts and offspring of the high apomixis father have a smaller diameter of the capitulum ($F_{1,125} = 11.0$, $p = 0.001$; $F_{1,124} = 5.3$, $p = 0.02$; Figure 5A, B). The diameter of the capitulum increases from the genotype Ah to Sl (Figure 5C; Ah to Al – $t = 2.7$, $p = 0.008$; Ah to Sh – $t = 3.2$, $p = 0.002$; Ah to Sl – $t = 4.2$, $p < 0.001$).

We conclude that the investment into floral display is associated with the mode of reproduction, but its magnitude depends on several loci in the genome.

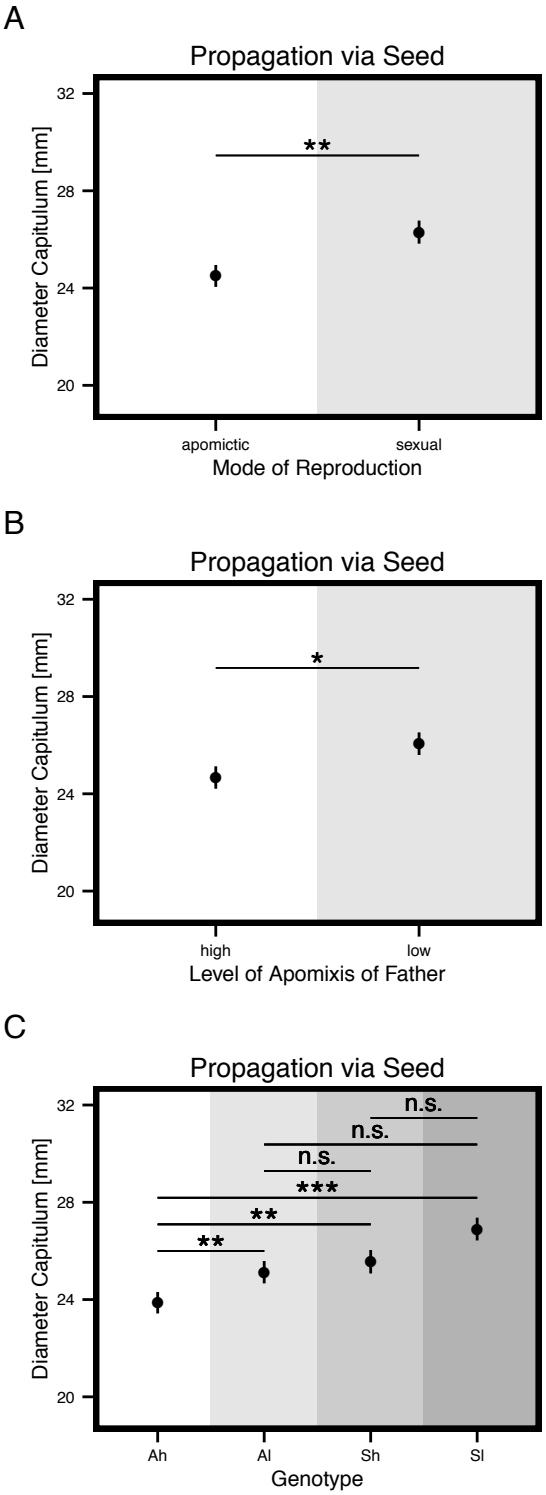


Figure 5. Propagation via seed – Diameter of capitulum is smaller in apomicts

A) Apomicts have a smaller diameter of the capitulum ($F_{1,125} = 11.0$, $p = 0.001$). **B)** Offspring of the low apomixis father have a smaller diameter of the capitulum ($F_{1,124} = 5.3$, $p = 0.020$). **C)** The diameter of the capitulum depends on the genotype of the plant ($F_{3,125} = 6.4$, $p < 0.001$).

grey background – different genotypes; Ah – Apomict, high apomixis father; Al – Apomict, low apomixis father; Sh – Sexual, high apomixis father; Sl – Sexual, low apomixis father; n.s. – not significant; $\cdot$ – $p < 0.1$; $*$ – $p < 0.05$; $**$ – $p < 0.01$; $***$ – $p < 0.001$

Darwinian Fitness

We refer to the proportion of reproducing plants to potential offspring as darwinian fitness. Darwinian fitness is a compound measure of fertility, germination rate (data not shown) and viability (data not shown).

Darwinian fitness is higher in offspring of the high apomixis father ($F_{1,157} = 9.77$, $p = 0.002$).

Apomicts have a more stable fitness with respect to the genotype of the father (interaction $F_{1,156} = 3.63$, $p = 0.059$, Figure 6A). The genotype Sh has the highest fitness. Genotype Sh has a higher fitness than Sl ($t = 3.32$, $p = 0.001$), and Ah ($t = 1.88$, $p = 0.062$), respectively (Figure 6B).

Therefore, we conclude that the level of fitness is regulated by the unrelated genotype. In addition, we conclude that apomixis provides stability, since the sexual genotypes differ with respect to the genotype of the father.

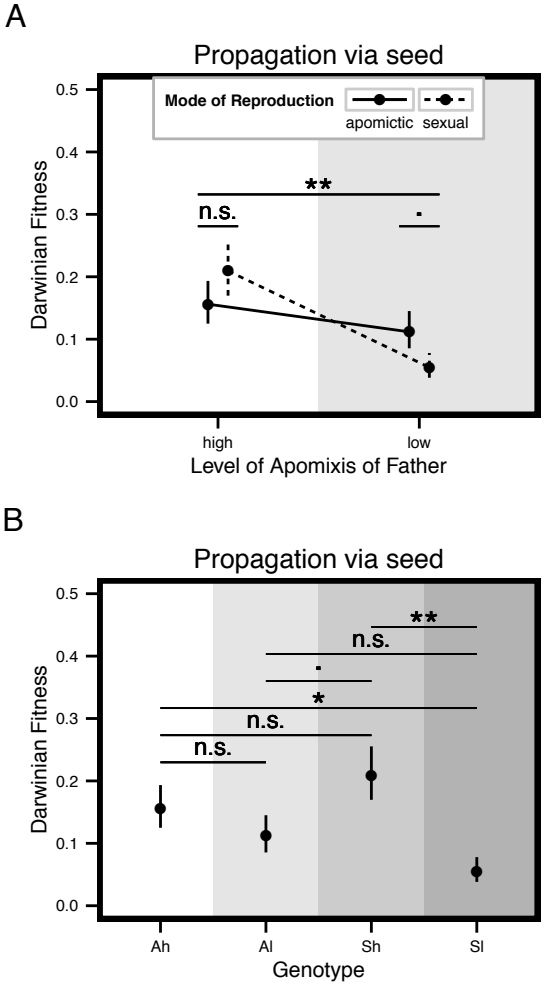


Figure 6. Darwinian fitness depends on the genotype

A) Offspring of the high apomixis father have a higher fitness than offspring of the low apomixis father ($F_{1,157} = 9.77$, $p = 0.002$). Apomicts have a more stable fitness with respect to the unrelated genotype (interaction $F_{1,156} = 3.63$, $p = 0.059$). **B)** Sh genotypes have the highest fitness, Sl genotypes the lowest. Sh genotypes have higher fitness than Sl genotypes ($t = 3.32$, $p = 0.001$), and as Ah genotypes ($t = 1.88$, $p = 0.062$).
Grey background – different genotypes; solid line – apomict; dashed line – sexual; Ah – Apomict, high apomixis father; Al – Apomict, low apomixis father; Sh – Sexual, high apomixis father; Sl – Sexual, low apomixis father; n.s. – not significant; · – $p < 0.1$; * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$

Summary of Results – Main Findings

We investigated the performance of apomictic and sexual siblings in different competition settings. Apomicts had a higher fertility in between-species competition and had a higher single seed mass under within-species competition. These are the only two settings, in which the mode of reproduction was the only explanatory factor. In all other cases, the mode of reproduction was not the only explanatory factor, as the unrelated genotype influenced competitiveness additionally.

For the number of ovules and the diameter of the capitulum, we found a general difference between apomictic and sexual siblings. However, the magnitude of these differences was determined by the unrelated genotype. In fact, both phenotypes were found to also generally differ between the two unrelated genotypes. Moreover, a general difference between the unrelated genotypes was found for only one phenotype, stem length.

Discussion

We aimed to disentangle the effects of the mode of reproduction from its unrelated genotype on competitiveness, by measuring fitness related phenotypes under different competition treatments.

Comparisons to the Previous Study of Sailer et al.

In contrast to a previous study (Sailer et al., submitted), we found no difference between apomictic and sexual siblings in biomass production under competition. However, “ancestral” sexual genotypes and invasive apomictic genotypes, both having undergone selection in a natural environment, were compared in the previous study, while in the current one, new genotypes, which have not been subjected to selection, were compared. Nevertheless do the new genotypes carry selected traits in their genomes, however in a new combination, since we used the invasive apomictic genotypes as fathers. The new genotypes have lost the trait of bigger growth. This result indicates that the intraspecific hybridization, which gave rise to the new genotypes, disrupted the beneficial allele combination of the father. This is in line with the destabilizing hybridization theory (Lynch 1984), which was also found in the previous study with newly created sexual pentaploid plants. In addition, this result hints towards a hidden cost of sex. We could disentangle the influence of the mode of reproduction from its unrelated genotype and conclude that the earlier findings were due to the unrelated genotype.

The same arguments are true for the the number of stolons produced. Additionally, we found that mix-cultures, which were genetically more diverse than mono-cultures, resisted between-species competition better. This result suggests that higher genetic diversity of a single species can increase resistance of a population to invasion by another species (Vellend 2006, Nitschke et al. 2010). Moreover, vegetative reproduction is the main reproductive pathway in natural populations (Winkler and Stöcklin 2002). We conclude that a high genetic diversity adds to the stability of a population.

Maximum stolon length was bigger in sexuals under within-species competition, which is the contrary to the finding of the previous study (Sailer et al., submitted). In addition, the unrelated genotype is of importance for the maximum stolon length. We conclude that maximum stolon length is linked to the unrelated genotype, and that it was co-selected with apomixis in the paternal line.

Overall, we conclude that the effects observed in the previous study were not solely due to the different modes of reproduction, but that the overall genotype was selected.

Apomixis is a trait, besides others, which was selected during the invasion process of *H. pilosella* in New Zealand.

Differences Between Apomictic and Sexual Siblings and the Influence of the Unrelated Genotype

Fertility is a trait which could be expected to be different between apomicts and sexuals, because autonomous apomicts do not rely on pollination (Koltunow and Grossniklaus 2003). We found fertility to be regulated by the genetic background. Nonetheless, apomicts exhibited a more stable fertility across the different competition treatments than sexuals. This is in line with the hypothesis of reproductive assurance (Darwin 1862, Stebbins 1957). Although fertility is regulated by the overall genotype, apomixis ensures a certain level of reproduction. As these results suggest, apomicts have reproductive assurance and do not need to find a mate for reproduction via seed. If no cross-pollination is necessary, the floral display (petals) is unnecessary for reproduction, and in an evolutionary context, is a structure which will eventually disappear (Darlington 1958). In fact, we found that apomicts had a smaller floral display than their sexual siblings. If selfing species are compared to closely related outcrossing ones, selfing species have smaller flowers, a phenomenon called selfing-syndrome (Ornduff 1969, Sicard and Lenhard 2011). We could show the selfing syndrome for the first time in apomictic and sexual siblings of a single species. The selfing syndrome was also described before in apomictic plants of *Ranunculus auricomus* (Steinbach and Gottsberger 1994, Hörandl 2008). In addition, the magnitude of the selfing syndrome was due to the overall genotype, which is consistent with QTL studies, which tried to map flower size (Sicard and Lenhard 2011).

Fertility was higher in apomicts in between-species competition, a setting which resembles the situation in a natural population. However, apomicts and sexuals had a different number of ovules, which in turn is regulated by the overall genotype. We conclude that the higher fertility of apomicts in between-species competition is partly due to the higher number of ovules and partly due to reproductive assurance.

Fertility is part of darwinian fitness, which we have defined as the proportion of reproducing plants to potential offspring. Darwinian fitness, computed as a compound measure of viability, fertility and germination rate, is regulated by the unrelated genotype. However, sexual half siblings differed in their darwinian fitness, while apomictic half siblings did not. We attribute this observation to the more stable fertility of apomicts, which is part of the darwinian fitness. We could not find a general difference in darwinian fitness

between apomicts and sexuals. Thus, we conclude that darwinian fitness is determined by the overall genotype, although apomixis can stabilize darwinian fitness by providing reproductive assurance of well adapted genotypes.

Conclusions

For all phenotypes in different competition settings, we found that the whole genome determines competitiveness. In all cases in which apomicts and sexuals differed, the unrelated genotype had an influence as well. Therefore, the mode of reproduction *per se* does not result in a fitness difference. Apomixis is facultative (Asker and Jerling 1992, Koltunow and Grossniklaus 2003, Tucker et al. 2003, Sailer et al., in preparation) and therefore enables a species to have reproductive assurance of adapted genotypes on the one hand, and creation of new genotypes, which can be selected if the environment changes, on the other. As a consequence of versatility of reproduction, species survival is more likely. Moreover, this versatility enables apomictic species to remove mutations via sexuality and create new apomictic and sexual genotypes. This results in an equilibrium between apomixis and sexuality, depending on mutational loads (Kondrashov 1985). This versatility and removal of mutations via sexuality might explain why populations of an apomictic species often consist of apomictic and sexual genotypes (van Dijk 2003, Hörandl and Paun 2007, Mráz et al. 2008). Taking all these arguments together, we might think of apomixis as an additional layer of diversity and variation.

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Dynamics of Apomixis and Sexuality on an Alpine Glacial Foreland

Dynamics of Apomixis and Sexuality on an Alpine Glacial Foreland

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Abstract

Apomixis, the asexual reproduction through seeds, is thought to be advantageous in sparse population densities and in colonization. This is due to the fact that apomicts do not need to find a mate to reproduce (Tomlinson's prediction), and a single individual would therefore be enough to found a new population (Baker's law). A certain level of sexuality would furthermore enable fast adaptation to new environments (Stebbins' prediction).

Here we present an observational field study of an apomictic species, *Hieracium pilosella* L., along a primary successional gradient in the Swiss alps. We have identified apomictic and sexual plants, their ploidy levels, fertility, and level of apomixis, to test if Tomlinson's, Baker's and Stebbins' predictions are true for single species and also applicable for apomixis.

We have found that levels of apomixis are low in general and that the level of apomictic offspring decreases with succession. Identification of two triploid populations of *H. pilosella* are attributable to a rare colonization event and therefore support Baker's law. The wide variation of the observed level of apomixis enables this species to continuously generate new genotypes by out- and in-crossing, as well as cloning successful genotypes by apomixis, which, according to Stebbins, is a property assigned to a successfully adapting species.

In summary, we show in a single species that Tomlinson's, Baker's and Stebbins' predictions for selfing also hold true for apomixis.

Introduction

Baker's law states that a single selfing individual is sufficient to found a new population (Baker 1955, 1967). Stebbins (1957) stated that plant populations adapted to certain types of temporary habitats possess a considerable selective advantage if they remain genetically constant across/throughout many generations, producing occasional burst of genetic variability after a disturbance. This is true for annual weeds, which have to build a new population frequently after disturbances. But it is also true for colonizers which are often subjected to new conditions. Individuals which are well adapted to the new condition are positively selected and due to selfing, the selected genotype(s) is(are) transmitted efficiently to the offspring (Stebbins 1957). A reproductive system which allows genetic constancy over many generations, but still enables outcrossing, favors adaptation to changing conditions (Stebbins 1957).

Tomlinson (1966) described the relationship between the mode of reproduction and reproductive success as a function of the chance of finding mates. The chance of finding a mate is crucial in sparse population densities, mainly during colonization of a new habitat or in succession. A selfing individual does not need to find a mate, therefore, selfing is advantageous in sparse population densities.

The same rationale can be transferred from selfers to apomicts. Apomixis is nowadays understood as the asexual reproduction through seeds (Asker and Jerling 1992). Several types of apomixis are distinguished. In sporophytic apomixis (adventitious embryony) the embryo develops from a sporophytic cell of the integument layer (sporophyte) of an ovule. The resulting seed contains the apomictic and the sexual embryo (Koltunow and Grossniklaus 2003).In gametophytic apomixis (diplospory and apospory) the embryo develops from a cell of the gametophytic tissue. In diplospory the embryo develops from the megaspore mother cell, while in apospory the embryo develops from an aposporous initial cell, which arises from a cell of the nucellus. In both cases, meiosis is avoided (apomeiosis) and embryo development is initiated without fertilization (parthenogenesis) (Asker and Jerling 1992, Koltunow and Grossniklaus 2003). In diplospory, recombination can still take place (Asker and Jerling 1992), leading to the so called autosegregation (van Dijk 2003).

The evolutionary consequences of selfing and apomixis are similar. A single apomict can found a new population and be successful as long as the habitat and enemy pressure are stable. Since every type of apomixis is facultative (Asker and Jerling 1992), apomicts have the potential to adapt to changing habitats, since new genotypes can be produced by

outcrossing. This can lead to bursts of genetic variability as described by Stebbins (Stebbins 1957).

We performed a field study on a glacial foreland to test if apomicts have an advantage in sparse population densities (early stages of succession) as predicted by Baker, Stebbins and Tomlinson (Baker 1955, Stebbins 1957, Tomlinson 1966).

Hieracium pilosella L. is an autonomous aposporous apomict which occurs along the succession of the glacial foreland of the Morteratsch glacier in the Upper Engadin in Switzerland, and therefore is an ideal model species for this study.

We sampled leaves for DNA-extraction and ploidy analysis, and seeds to analyze fertility and the mode of reproduction. Here, we present our findings on the dynamics of apomixis in the field and show that the predictions made by Baker, Stebbins and Tomlinson hold true for apomicts.

Materials and Methods

Study species

Hieracium pilosella L. is a self-incompatible, perennial, monocarpic, herbaceous species including sexual and apomictic lineages (autonomous apospory) with different ploidy levels. *H. pilosella* usually grows in patches of individual plants (vegetative reproduction via aboveground stolons). It occurs in ploidy levels from 3C to 8C (1C = haploid genome) (Greilhuber et al. 2005, Mráz et al. 2008).

Study site

The Morteratsch glacial foreland in the Upper Engadin in south-eastern Switzerland is a very well suited site to study primary succession. There is data on isochrones (a line on a chart or map connecting points (localities) at which a given event occurs simultaneously (Lincoln et al. 1998)) of deglaciation dating back to 1857 when the Morteratsch glacier had its biggest extent ("Glaciological reports (1881-2011) 'The Swiss Glaciers'" 2012). The altitude difference between the end moraine and todays glacier front is minimal (< 200m).

Sampling

As a first step the whole glacial foreland was searched for occurrence of *H. pilosella* and their positions were marked with GPS (GPSmap 60CS, Garmin, Garching, Germany) to an accuracy of 5 m. The positions were transferred to the topographical Swiss map (Topo Schweiz V1, Garmin, Garching, Germany) using the MapSource software (Garmin, Garching, Germany). The map with the marked positions was printed and the isochrone data was constructed based on a published map (Burga et al. 2010). Patches lying on the isochrones were dismissed. The glacial foreland was split in six twenty-year time windows. Ten patches of *H. pilosella* on each side of the river were randomly selected for sampling. Furthermore, 3 individuals from the centre of a patch and 3 individuals from the edges of a patch were collected and the maximum number was 6 plants per patch. DNA and seeds were collected from 234 plants coming from 74 patches.

In July 2011, after flowering of *H. pilosella*, the two youngest leaves from each plant were sampled. One leaf was shock-frozen in a vapor-shipper (SC 4/2 V, MVE Biomedical, Georgia, USA). The tip of the second leaf was placed in a 1.2 ml cluster tube (Thermo Scientific, Wohlen, Switzerland) containing 50 μL of mQ water (18 MΩ) and one 3 mm stainless steel bead (Schieritz & Hauenstein AG, Zwingen, Switzerland) and stored in a

cooling bag. Closed capitula were bagged using individually marked tea filters. In August 2011, the individually marked tea filters containing the seeds were collected and placed in plastic containers containing silica gel to ensure fast drying of the seed material. Seeds were stored at 4°C, 30% humidity until used.

DNA-extraction

DNA was extracted using the DNeasy Plant Mini kit (Qiagen) following the manufacturers instructions. Samples were eluted in 2 x 50 µL AE buffer.

Ploidy Analysis

The ploidy level of the collected plants was determined within 48 h after collection by ploidy analysis following the two-step method described by Dolezel and colleagues (2007) with minor modifications. A small piece of a *Bellis perennis* (1.72 pg DNA per nucleus) leaf was added to the collected leaf material, which was in 50 µL water, as internal standard. 50 µL of 0.2 M citric acid (Fluka, Buchs, Switzerland), 0.01% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) was added to a total volume of 100 µL and the leaf material was disrupted by shaking it 2 times for 30 sec at 30 Hz using a mixer-mill (MM300, Retsch, Haan, Germany). After bead-beating 100 µL of 0.1 M citric acid (Fluka, Buchs, Switzerland), 1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) were added and mixed by inverting the plates to achieve a concentration of 0.1 M citric acid and ca. 0.5% Triton-X-100 in a total volume of 200 µL.

The solution was filtered through fritted deep well plates (Nunc, Thermo Scientific, Wohlen, Switzerland) into 96-well V-bottom plates (Sarstedt, Numbrecht, Germany). Nuclei were collected by centrifugation at 150 g for 5 min at 20°C (Centrifuge 5810R, Eppendorf, Schönebuch, Switzerland). The supernatant was removed and nuclei were resuspended in 40 µL 0.1 M citric acid, 0.5% Triton X-100. 160 µL of staining solution (0.4 M Na_2HPO_4, Merck, Darmstadt, Germany; 5.5 µg/mL DAPI, 4',6-diamidino-2-phenylindole, Invitrogen, Eugene, Oregon; 0.2 µL/mL 2-mercaptoethanol, Sigma-Aldrich, Steinheim, Germany) were added 2 min prior to analysis by the flow cytometer robotics (Quanta SC MPL, Beckman-Coulter, Nyon, Switzerland). The run was stopped at a count of 6000 in the defined sample region or latest after 3:40 min runtime.

The haploid (1C) DNA content of *Bellis perennis* and *Hieracium pilosella* is the same (Suda et al. 2007). To determine the ploidy of *Hieracium* the median of the *Hieracium* value was divided by the median of the *Bellis* value and multiplied by 2, to account for diploidy of the *Bellis* internal standard.

Fertility

Seeds and empty seeds (seed coat) were sorted and counted manually. Both were weighted separately on an analytical scale to a precision of 0.1 mg (AM50, precision = 0.1 mg, Mettler, Greifensee, Switzerland).

Flow Cytometric Seed Screen

Single seeds were put into 1.2 mL cluster tubes (Thermo Scientific, Wohlen, Switzerland) containing one 3 mm stainless steel bead (Schieritz & Hauenstein AG, Zwingen, Switzerland). 80 µL of 0.1 M citric acid (Fluka, Buchs, Switzerland), 0.1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) were added. Seeds were disrupted by shaking them 2 times for 3 min at 30 Hz in a mixer mill (MM300, Retsch, Haan, Germany). Disruption was visually controlled. If the disruption was not optimal, seeds were shaken for another 3 min at 30 Hz. After disruption, 80 µL of 0.1 M citric acid (Fluka, Buchs, Switzerland), 1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) were added to a total volume of 160 µL. The solutions were mixed by inverting the plates 40 times.

The solution was filtered through fritted deep well plates (Nunc, Thermo Scientific, Wohlen, Switzerland) into 96-well V-bottom plates (Sarstedt, Numbrecht, Germany). Nuclei were collected by centrifugation at 150 g for 5 min at 20°C (Centrifuge 5810R, Eppendorf, Schönebuch, Switzerland). The supernatant was discarded.

The internal standard was produced separately from *Bellis perennis* seeds by crushing the seeds using a pistil and mortar. About 100 seeds were used for two plates. The crushed seeds were mixed with 500 µL 0.1 M citric acid (Fluka, Buchs, Switzerland), 0.5% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) and the solution was filtered through 30 µm filters (CellTricsTM, Partec) into 1.5 mL Eppendorf tubes (Sarstedt, Numbrecht, Germany). Nuclei were collected by centrifugation at 200 g for 5 min at room temperature (Centrifuge 5415D, Eppendorf, Schönebuch, Switzerland). The supernatant was removed and *Bellis* nuclei were resuspended in 6.5 mL 0.1 M citric acid, 0.5% Triton X-100. The *Hieracium* nuclei were resuspended in 30 µL of the *Bellis* nuclei solution.

80 µL of staining solution (0.4 M Na_2HPO_4, Merck, Darmstadt, Germany; 5.5 µg/mL DAPI ,4',6-diamidino-2-phenylindole, Invitrogen, Eugene, Oregon; 0.2 µL/mL 2-mercaptoethanol, Sigma-Aldrich, Steinheim, Germany) were added to a total volume of 110 µL 2 min prior to analysis by the flow cytometer robotics (Quanta SC MPL, Beckman-Coulter, Nyon, Switzerland).

Ploidy of the embryo/endosperm was calculated by dividing the median of embryo/endosperm by the median of *Bellis* and multiplication by 2.

We screened up to 12 seeds per plant. This enabled us to detect as low as 8% apomixis per plant. Plants scored as sexual have therefore less than 8% apomixis.

Statistical Analysis

Developmental Origin of Seeds

To determine the developmental origin of the collected seeds, linear discriminant analysis was employed. As training set we used data from histograms of a flow cytometric seed screen (see above) from another experiment which could be clearly assigned to one of the 4 types (n + 0, n + n, 2n + 0, 2n + n, following the nomenclature of Harlan and deWet 1975) of offspring of an apomictic plant. In sexual seeds (n + n) the ratio between endosperm and embryo is 1.5. In 2n + 0 seeds the endosperm to embryo ratio is 2. Furthermore, ploidy of the seeds decrease compared to the mother plant in n + 0 offspring, and increase in 2n + n offspring. This ploidy difference of the embryo to the mother and the ratio between the ploidy of the endosperm and the embryo were used as discriminators. The linear discriminator function had less than 2% incorrect assignments on the training set.

Only datasets with an HPCV < 5% (Half Peak Coefficient of Variance) were used for the ploidy of the mother, and HPCV < 7% for the the ploidy of the embryo were taken into the analysis. We used a higher HPCV value as cutoff in the seed screen, since the histograms from seeds from the field are noisier than the histograms from leaves. The final dataset contained 158 individual plants.

The assignment graph revealed that no n + 0 offspring was recovered at Morteratsch and that some 2n + 0 were wrongly assigned as n + 0. Nonetheless, a separation of apomictic (n + 0, 2n + 0, 2n + n) from sexual (n + n) offspring was possible.

Analysis of Variables

Six dependent variables and 5 explanatory variables were tested. Two dependent variables were also used as explanatory ones in some cases as follows.

The following variables were tested 1) abundance of apomicts, 2) level of apomixis, 3) level of apomictic offspring, 4) ploidy of the mother plant, 5) fertility and 6) offspring mass. The explanatory variables were i) succession, ii) mode of reproduction, iii) position in patch, iv) ploidy of mother plant and v) fertility.

Generalized linear models (glm) were fitted in the order of intrinsic variables (e.g. ploidy mother plant) first, followed by environmental (extrinsic) variables (e.g. succession). Backward elimination of non-significant terms was employed, with keeping variables if they were part of significant interactions, to arrive at the final model.

In case of proportions (binary count data: abundance, level of apomixis, level of apomictic offspring, fertility) variance was modeled by using the family function “binomial”. In case of over- or underdispersion of the data the family function “quasibinomial” was used to model variance. To model means, the default link-function “logit” was used.

In case of continuous data (ploidy of mother plant, offspring mass) the family function “gamma” was used to model variance with the default link function “inverse” for modeling means.

Significance was tested using Analysis of Deviance (ANODEV) using the Chi-Square test. If the data was over- or underdispersed the F-test was used instead.

Abundance

Successional change of abundance of apomictic plants was tested for.

Level of Apomixis

The level of apomixis is the proportion of seeds which developed apomictically.

Level of Apomictic Offspring

The number of apomictic offspring was estimated by multiplying the number of seeds by the level of apomixis and rounding it to the next integer. To calculate the level of apomictic offspring we multiplied fertility by the level of apomixis.

Fertility

Fertility is the proportion of ovules which developed into seeds. The number of ovules is the number of seeds plus the number of seed coats.

Offspring Mass

Offspring mass was computed by subtracting the single seed coat mass from the single seed mass.

All statistical analyses were carried out in R (R Developmental Core Team 2010). Graphs were produced using the ggplot2 package (Wickham 2009) and the grid package (Murrell 2005).

Results

The abundance of apomictic plants does not change along succession ($F_{1,4}$ = 3.9, p = 0.121). Apomicts occur everywhere along succession with the same abundance (Figure 1A).

Succession does not influence the level of apomixis (proportion of seeds which are of apomictic origin). The level of apomixis is dependent on the ploidy of the mother plant only ($F_{1,73}$ = 37.6, p < 0.001). Hexaploid plants show a big variation in the level of apomixis (Figure 1B).

Ploidy of the mother plants changes along succession differently for apomicts and sexuals (interaction $F_{1,154}$ = 15.7, p < 0.001). Ploidy of sexuals decreases ($F_{1,81}$ = 4.3, p = 0.040), while the ploidy of apomicts increases ($F_{1,73}$ = 11.3 , p = 0.001). Plants with low ploidy only occur in the early stages of succession and all are apomictic (Figure 1C).

Apomicts have a slightly higher fertility than sexuals ($F_{1,156}$ = 2.9, p = 0.090; Figure 1D). Furthermore, the mode of reproduction and ploidy of the mother plant show an interaction in Analysis of Deviance (ANODEV, interaction $F_{1,154}$ = 3.20 , p = 0.076), which is due to the fact that sexuals of low ploidy do not occur in the Morteratsch glacial foreland (Figure 1C).

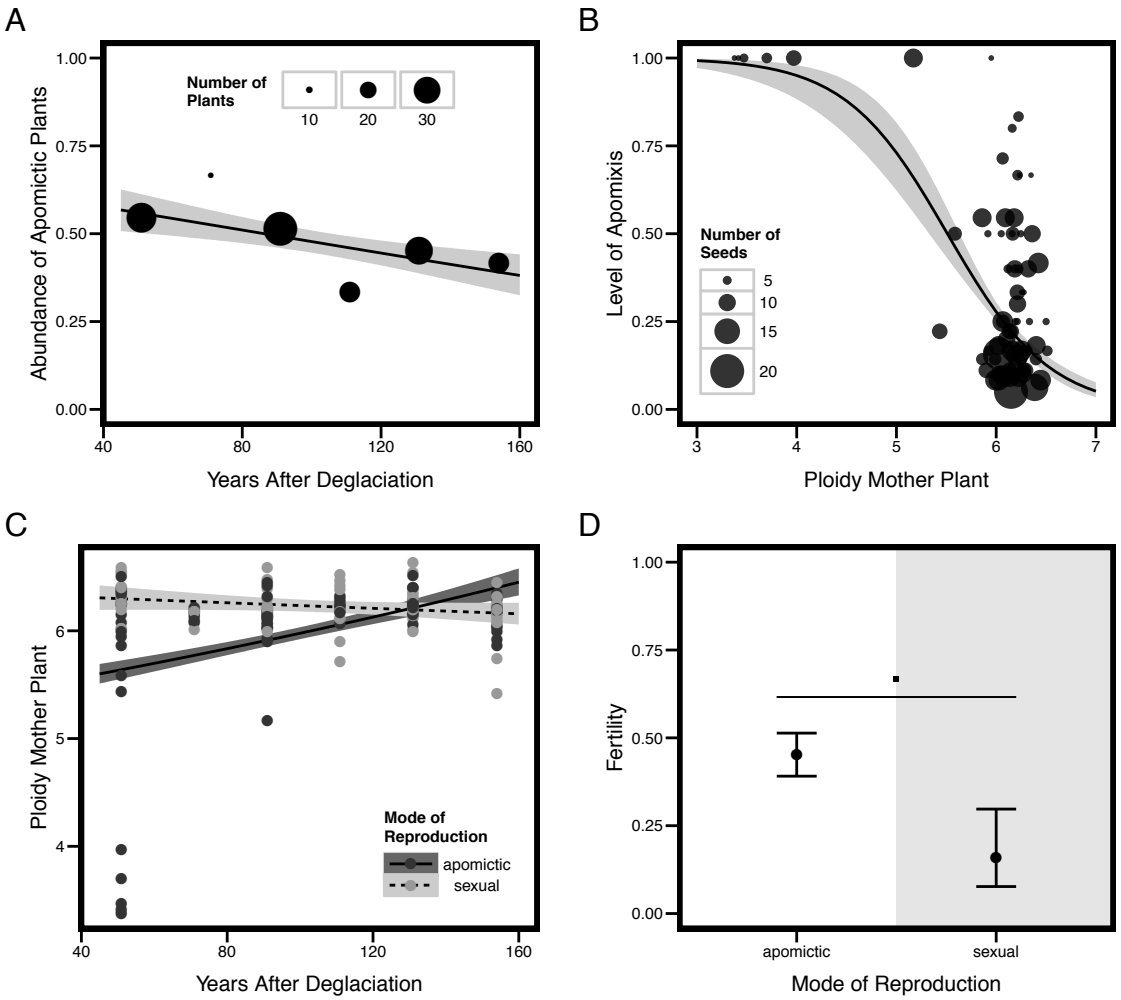

Figure 1. Abundance of apomicts, level of apomixis, ploidy of mother plants and fertility of apomicts and sexuals

A) Abundance of apomicts does not change along succession ($F_{1,4}$ = 3.9, p = 0.121). Diameter of dots is proportional to the number of plants. **B)** The level of apomixis depends on the ploidy of the mother plant ($F_{1,73}$ = 37.6, p < 0.001). Hexaploid plants vary vastly for the level of apomixis. Diameter of dots is proportional to the number of seeds analyzed per plant. **C)** Ploidy of mother plants changes differently for apomicts and sexuals (interaction $F_{1,154}$ = 15.7, p < 0.001). Dark grey – apomicts, light grey – sexuals **D)** Fertility is higher in apomicts than in sexuals ($F_{1,156}$ = 2.9, p = 0.090). Grey background – sexual plants
Lines depict the generalized linear model. Shaded areas are ±1 sem. · – p < 0.1

Fertility is not accounted for in the level of apomixis. To account for different fertility the level of apomictic offspring was computed. The level of apomictic offspring is the proportion of ovules which developed apomictically into mature seeds. The level of apomictic offspring depends on the ploidy of the mother plant and on succession. Both factors interact ($F_{1,152}$ = 15.5, p < 0.001; Table 1). This is due to the dependency of the level of apomixis on the ploidy of the mother plant, which is changing along succession. Additionally, the level of apomictic offspring is higher if the plant did not grow in a patch ($F_{2,154}$ = 3.4, p = 0.037; Table 1).

Table 1: Analysis of Deviance for the Level of Apomictic Offspring.
ANODEV was performed on a generalized linear model using the F-test due to overdispersion of the data. The level of apomictic offspring is defined by intrinsic as well as extrinsic factors.

	Df	Residual Df	F	Pr(>F)
NULL	NA	157	NA	NA
Ploidy Mother	1	156	89.159	0.0000
Position	2	154	3.372	0.0369
Succession	1	153	4.458	0.0364
Ploidy Mother : Succession	1	152	15.501	0.0001

Offspring mass is the single shell mass subtracted from single seed mass. Offspring mass decreases with increasing fertility, depending on succession (interaction $F_{1,153} = 3.1$, $p = 0.79$; Table 2). Offspring mass increases with increasing ploidy ($F_{1,155} = 11.3$, $p = 0.003$; Table 2).

Table 2: Analysis of Deviance for Offspring Mass.
ANODEV was performed on a generalized linear model using the F-test due to underdispersion of the data. Offspring is defined by intrinsic as well as extrinsic factors.

	Df	Residual Df	F	Pr(>F)
NULL	NA	157	NA	NA
Reproduction	1	156	0.198	0.6572
Ploidy Mother	1	155	9.786	0.0021
Succession	1	154	2.975	0.0866
Reproduction : Succession	1	153	3.704	0.0561

Discussion

From Tomlinson's model (Tomlinson 1966) we would predict different abundances of apomicts and sexuals in different stages of succession. We did not find this relationship, probably due to the resolution of successional stages, which might not have been high enough. Using the distance of the patch to the glacier front instead of successional stages to increase resolution did not change this result.

Considering the amount of apomictic offspring, we found a decline with succession. In addition, it is higher in plants which grow sparsely. Both findings support Tomlinson's model (Tomlinson 1966). In turn, the amount of apomictic offspring depends on the ploidy of the mother. Triploid plants have the highest level of apomictic offspring. Uneven ploidy leads to unbalanced chromosome segregation in meiosis, which results in non-functional meiotic products. Avoidance of meiosis, as it happens in apomixis, offers a possibility to produce functional meiotic products, which develop into functional gametophytes (escape from sterility, Darlington 1958). As a consequence, seeds from plants with uneven ploidy should be of apomictic origin. Our result fits this expectation.

Most plants are hexaploid at the glacial foreland of Morteratsch. Triploids are likely to be the n + 0 offspring (following the nomenclature used in Harlan and deWet 1975) of hexaploids. We were not able to recover n + 0 offspring in our samples, but as Baker's law states, a single individual would suffice to found a new population. We consider the occurrence of triploid plants to be the result of rare n + 0 offspring. In fact, there is a bias against n + 0 offspring (Sailer et al., unpublished). Furthermore, the occurrence of triploids is limited to the early stages of succession, which indicates a successful colonization event in sparse population densities, supporting Baker's law (Baker 1955).

The limitation of triploids to early successional stages might be due to lower vegetative competitiveness of triploid plants, which grow less vigorous than plants of higher ploidy (Sailer et al., submitted).

Apomicts showed a higher fertility than sexuals. This difference could be caused by pollen limitation (Galen 1985, Muñoz and Arroyo 2006, De Cauwer et al. 2010). The same difference was also observed under no pollen limitation in a different experiment (Sailer et al., in preparation), however, apomicts had more ovules than sexuals and the authors concluded that the different fertility is the result of the different number of ovules. We therefore conclude that the difference in fertility in the natural population is due to pollen limitation.

The higher fertility of apomicts results in a higher propagule pressure. Propagule pressure is a composite measure of the number of individuals released into a region to which they are not native (Carlton n.d.). This trait is part of the PAB model (Propagule pressure, Abiotic & Biotic characteristics) of invasion (Catford et al. 2009). A high propagule pressure enables colonization and establishment not only in invasion, but also in succession. We suggest that residual sexuality of apomicts and the possible outcrossing of apomixis gave rise to new apomictic genotypes, which are adapted to a new habitat. We interpret this finding in the way that apomicts are genetically diverse and are adapted to different habitats. This is in line with Stebbins (1957).

However, high fertility results in lower offspring mass, which is a major trade-off in nature (Smith and Fretwell 1974). In turn, lower offspring mass results in lower germination rate (Sailer et al., in preparation). We suggest that this trade-off is counteracting the higher fertility of apomicts.

Overall, we found mainly low levels of apomixis. Contrasting with our study, high levels of apomixis were found in an invasive range in New Zealand (Houliston and Chapman 2004). However, the the main cytotype was an apomictic pentaploid (Chapman et al. 2000, Houliston and Chapman 2004). The level of apomixis is also high in aneuploid plants (Okada et al. 2007). In both the pentaploid and the aneuploid plants, the high level of apomixis might be due to selection for an escape from sterility (Darlington 1958). If alpine plants in sparse population densities are considered, apomixis was found to be rare even in apomictic species. For example, only 1 of 14 apomictic species produced seeds of apomictic origin in the subnival to nival area of the alps (Hörandl et al. 2011). We suggest that plants of even ploidy levels are not under selection for apomixis because there is no pressure to escape from sterility (Darlington 1958). It is more likely that apomixis provides an additional possibility for reproduction and spread. Apomixis is a trait which is in a dynamic equilibrium with its related trait of sexuality (Kondrashov 1985). This might explain the general low levels of apomixis found and the big variation in the level of apomixis.

In summary, we found support for Baker's law, Stebbins' and Tomlinson's predictions and that those hold true for apomicts in a field study concerning a single species. In addition we have shown that apomixis is facultative in a natural alpine population, and that the level of apomixis is low.

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Transgenerational Phenotypic Stability in Apomictic Clones

Transgenerational Phenotypic Stability in Apomictic Clones

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Abstract

Heterotic F1 hybrids played an important role in the green revolution. By using dwarfed varieties, improved agricultural practice with fertilizers and pesticides, plus heterotic hybrids, the yields were dramatically increased. The big disadvantage of F1 hybrids, however, is segregation and loss of heterosis in subsequent generations. Consequently, heterotic hybrids have to be generated anew for every growing season.

Fixation of heterosis by apomixis, the asexual reproduction through seeds, has been proposed as a solution since the 1930s. But so far, all attempts to introgress apomictic relatives into crop plants have failed. However, given that the heterosis effect may not be of purely genetic nature, it is not known whether the fixation of heterosis by apomixis is possible. To address this question whether phenotypes are stably inherited over generations in apomictic lineages, we use *Hieracium pilosella* L. as an apomictic model system.

H. pilosella is an autonomous aposporous apomict. Apospory results in offspring with completely identical genetic constitution in *cis* as the mother, a requirement for fixation of complex genotypes, and presumably also of heterosis. Here we show the results of a pilot experiment using interspecific hybrids and the outline of an experiment using intraspecific

hybrids. For the latter, we generated new apomictic lines with varying degrees of apomixis and propagated them apomictically (clonally) for two generations.

At time of defense of this thesis, the analysis of this experiment is still pending. This is mainly due to the long generation time of 6-8 months of *H. pilosella.* The analysis will be finished after this PhD thesis has been completed.

The results of the intra-species hybrid experiment will reveal whether fixation of heterosis by apomixis is possible as is generally assumed. Furthermore, we will learn more about the inheritance, ecology and evolutionary success of apomicts.

Introduction

Heterosis is the greater vigor of growth, survival, and fertility in hybrids than in the parents (Chen 2010). Heterotic F1 hybrids are essential to maintain and increase yield in many agricultural crop productions. The generation of F1 hybrids includes crossing of parental inbred lines and extensive phenotyping to select the best performing genotypes. This procedure is expensive and time-consuming. Furthermore, hybrid seeds have to be generated anew for every growing season, since heterosis is lost in the F2 generation, due to segregation. This leads to dependencies upon seed producers.

The long-term fixation of hybrid vigor would enable poor farmers to keep their own seeds and would tremendously reduce the cost for F1 hybrid seed production, since the generative cross and phenotyping have to be done only once. Fixation of hybrid vigor is therefore seen as the holy grail of agriculture as it would be beneficial for both breeders and farmers (Spillane et al. 2004). Apomixis, the asexual reproduction through seeds, can fix any – however complex – genotype and is therefore thought to enable the fixation of heterosis. While this is possible theoretically, all attempts to introgress apomictic traits from related species into crops failed (Spillane and Steimer 2001). As a result, it is not known whether the heterotic phenotype can indeed be fixed across generations by apomixis.

Inbred lines that are used for production of heterotic hybrids are nearly isogenic (the same alleles in all loci), and therefore phenotypically stable. The molecular and genetic basis of heterosis is, however, not fully understood and although much of the effect may depend on the genotypes of the parental lines, there may also be a considerable epigenetic contribution. Indeed, epigenetic effects in *Arabidopsis thaliana* hybrids have been described (Johannes et al. 2009, Chen 2010, Greaves et al. 2012, Fujimoto et al. 2012). Heterotic hybrids show molecular changes that differ from the expected Mid Parent Value (MPV), which is a measure of heterosis. In *Arabidopsis thaliana* hybrids such non-additive effects concern gene expression, DNA-methylation, and the expression of 24 nt siRNAs (Greaves et al. 2012). These epigenetic changes are associated with higher photosynthetic activity (Fujimoto et al. 2012), which is a basis for higher yields.

Should the heterosis effect indeed depend in part on an epigenetic effect or on parental factors, apomictically (clonally) propagated, heterotic hybrids may not be phenotypically stable over generations. Using *Hieracium pilosella* L., an autonomous aposporous apomict, as a model organism, we are able to study phenotypes over apomictic generations (Koltunow et al. 1998). Since apomixis deregulates the sexual processes in space and time, it is a facultative trait (Asker and Jerling 1992, Bicknell et al.

2003, Bicknell and Koltunow 2004, Houliston and Chapman 2004). The facultative nature of apomixis enables the generation of new apomictic lines via outcrossing.

First, we present the results of the pilot study with interspecific hybrids. Second, we present results of differing phenotypes between apomictic and sexual siblings as well as results on apomictic fertility of generation A1.

Materials and Methods

Plant Species

Hieracium pilosella L. is a self-incompatible, perennial, monocarpic herbaceous species including sexual and apomictic lineages, which can occur at different ploidy levels. Plants can reproduce vegetatively via aboveground stolons. Apomictic lineages are of the autonomous apospory type, meaning that neither the embryo (parthenogenesis) nor the endosperm (autonomy) need fertilization to trigger development.

Two loci are responsible for apomixis in *H. pilosella*: *Loss Of Apomeiosis* 1 (*LOA1*) and *Loss Of Parthenogenesis* (*LOP1*) (Catanach et al. 2006). *LOA1* plants are apomeiotic, *LOP1* plants are parthenogenetic. Segregation of the two loci results in 4 different offspring types: 1) n + n, *loa1/lop1*, sexual, 2) n + 0, *loa1/LOP1*, polyhaploids, 3) 2n + 0, *LOA1/LOP1*, maternal, and 4) 2n + n, *LOA1/lop1*, B_{III}-hybrids (following notation of Harlan and deWet 1975).

Generation of Apomictic Lines

Four sexual hexaploid lines (sP6, sexual Pilosella 6-ploid) were isolated from two populations from the Morteratsch glacial foreland, Upper Engadin, Switzerland (MoK5-4: 791849, 145561; MoG20-2, MoG20-8, MoG23-8: 792087, 148071; GPS, Swiss Grid). Two apomictic hexaploid lines (aP6, apomictic Pilosella 6-ploid) were isolated from two populations in New Zealand (line LaP1, Lake Pukaki, latitude: -44.15848, longitude: 170.22020 and line MwR1, Molesworth Road, latitude: -42.00933, longitude: 172.95406). Line LaP1 had low apomictic fertility (low), while line MwR1 had high apomictic fertility (high).

Both aP6 lines were crossed onto the four sP6 lines, resulting in 7 different families, since one cross could not be made. The F1 of these crosses were grown in the greenhouse and tested for apomixis by decapitation (Koltunow et al. 1995). Decapitation identified parthenogenetic plants. In A1 (apomictic generation 1), ploidy of plants was tested to identify n + 0 offspring. Lines identified as 2n + 0 offspring were propagated to A2.

Propagation and Growth Conditions

Seeds were surface sterilized and germinated in petri dishes on half strength MS-medium (Murashige and Skoog 1962) (containing MS salts (Carolina, Burlington, North Carolina), Sucrose (Applichem, Darmstadt, Germany) and Phytoagar (Gibco BRL, Paisley,

Scotland)) in a Percival Scientific climatic cabinet (CU-36L6/D, CLF Plant Climatics GmbH, Wertingen, Germany) at 22°C/18°C (day/night) 14h light and 10h dark cycle after 72h stratification at 4°C. Germination was counted every day for 7 days.

Seedlings were transferred to a nutrient-poor soil ("Dachgartenerde extensiv", Ricoter Erdaufbereitungs AG, Frauenfeld, Switzerland), to mimic the field situation, in pots of 1.3 L of volume when they had produced two to three true leaves. Pots were filled will 700 g of soil covered with 200 g of sand. Seedlings were grown in the greenhouse for three days under a humidor after being transferred to soil. Watering was automated.

Ploidy Analysis by Flow Cytometry

The ploidy level was determined by ploidy analysis following the two-step method described by Dolezel and colleagues (2007) with minor modifications. One young *H. pilosella* leaf was placed into a 1.2 mL cluster tube (Thermo Scientific, Wohlen, Switzerland), which contained one 3 mm stainless steel bead (Schieritz & Hauenstein AG, Zwingen, Switzerland). A small piece of a *Bellis perennis* (1.72 pg DNA per nucleus) leaf was added to the leaf of *H. pilosella* lines as internal standard. 80 µL of 0.1 M citric acid (Fluka, Buchs, Switzerland), 0.01% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) were added and the leaf material was disrupted by shaking it 2 times for 30 sec at 30 Hz using a mixer-mill (MM300, Retsch, Haan, Germany). After bead-beating 80 µL of 0.1 M citric acid (Fluka, Buchs, Switzerland), 1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) were added and mixed by inverting the plates to achieve a concentration of 0.1 M citric acid and ca. 0.5% Triton-X-100 in a total volume of 160 µL.

The solution was filtered through fritted deep well plates (Nunc, Thermo Scientific, Wohlen, Switzerland) into 96-well V-bottom plates (Sarstedt, Numbrecht, Germany). Nuclei were collected by centrifugation at 150 g for 5 min at 20°C (Centrifuge 5810R, Eppendorf, Schönebuch, Switzerland). The supernatant was removed and nuclei were resuspended in 40 µL 0.1 M citric acid, 0.5% Triton X-100. 160 µL of staining solution (0.4 M Na_2HPO_4, Merck, Darmstadt, Germany; 5.5 µg/mL DAPI, 4',6-diamidino-2-phenylindole, Invitrogen, Eugene, Oregon; 0.2 µL/mL 2-mercaptoethanol, Sigma-Aldrich, Steinheim, Germany) were added 2 min prior to analysis by the flow cytometer robotics (Quanta SC MPL, Beckman-Coulter, Nyon, Switzerland). The run was stopped at a count of 6000 in the defined sample region or latest after 3:40 min runtime.

The haploid (1C) DNA content of *Bellis perennis* and *Hieracium pilosella* is the same (Suda et al. 2007). To determine the ploidy of *Hieracium* the median of the *Hieracium*

value was divided by the median of the *Bellis* value and multiplied by 2, to account for diploidy of the *Bellis* internal standard.

Experimental Design

The experiment is outlined in figure 1. Crossings generated 51 different apomictic lines originating from 7 families. Seeds from decapitated capitula of 26 different lines could be harvested and propagated to the next generation. No apomictic seeds could be used from 3 families, since there were too few apomictic seeds produced, or the plants flowered very late. Harvested seeds were germinated, building generation A1. Ploidy of A1 plants was determined by flow cytometry to identify n + 0, and 2n + 0 offspring (Harlan and deWet 1975). Seeds from decapitated capitula of 2n + 0 plants were harvested. Generation A2 consisted of 19 different lines from 3 different families. Lines were lost due to bad germination rates. The selected 19 lines plus the apomictic paternal lines were grown from generation A1 and A2 at the same time in a fully randomized design in a greenhouse chamber with automated watering and 16h light/ 8h dark cycle and 20°C day/ 18°C night. Ploidy of all plants was determined by flow cytometry to identify possible n + 0 offspring. Each line was replicated 8 times per generation, if possible, otherwise at least 3 times. Each generation, one young leaf was sampled for DNA-extraction. Leaves are stored at -80°C until extraction.

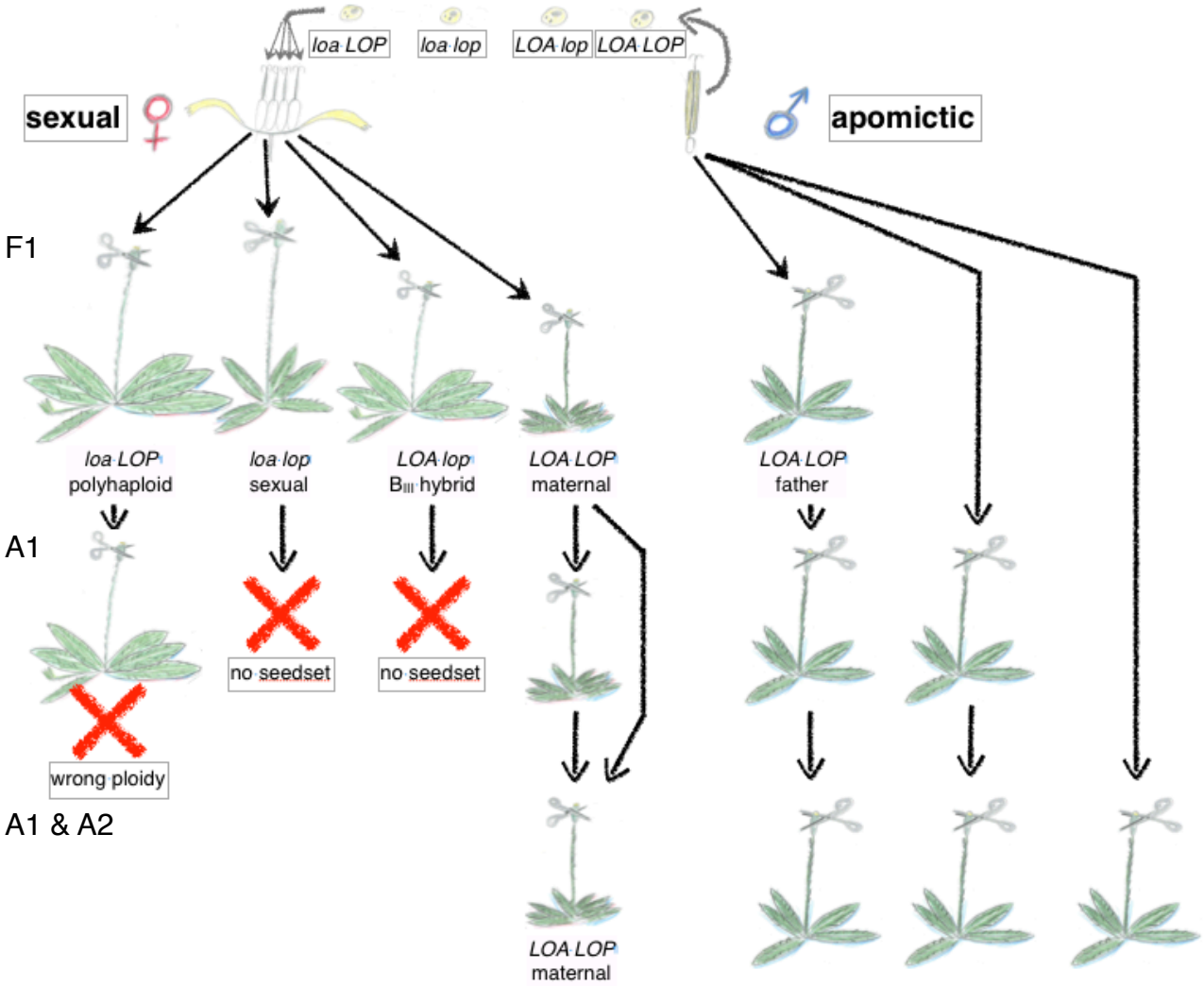


Figure 1. Experimental Design

Outline of the experiment. The male gametophyte is not affected by apomixis. Therefore the two responsible loci *LOA* and *LOP* can segregate independently. **F1)** The F1 offspring consist of all four possible combinations, which are depicted. F1 plants are tested for apomixis by decapitation. If *LOP* is absent, no seeds will be set after decapitation. **A1)** In generation A1 ploidy is measured to identify plants in which *LOA* is absent. These plants will have half the ploidy of the mother. Only if both *LOA* and *LOP* are present, the A1 plant will be a maternal clone. **A1 & A2)** Maternal clonal plants of generations A1 and A2 are grown at the same time in a fully randomized design with 3-8 replicates. The apomictic fathers of different apomictic generations are also grown as a control at the same time.

Phenotyping

The following phenotypes of A1 and A2 plants were measured and will be analyzed for differences between two apomictic generations.

1) Diameter of rosette at bolting
2) Diameter of rosette at flowering
3) Number of leaves at bolting
4) Number of leaves at flowering
5) Age at bolting
6) Age at flowering

7) Age at seed set
8) Diameter of capitulum at first day of flowering
9) Length of stem at seed set
10) Number of seeds
11) Number of empty seed shells
12) Number of ovules (number of sees + number of empty seed shells)
13) Apomictic fertility (number of seeds / number of ovules)
14) Mass of seeds
15) Mass of empty seed shells
16) Mean seed mass
17) Fecundity (number of flowering plants / number of seedlings)
18) Germination rate (number of germinated seeds / number of seeds plated)
19) Darwinian fitness (1 generation; germination rate * fecundity * fertility)

Statistical Analysis

Apomictic fertility, number of ovules and single seed mass from the pilot experiment were analyzed using analysis of deviance (ANODEV) of a generalized linear model (GLM). In case of over- or underdispersion of the data, the F-test, otherwise the Chi-square test was used. For apomictic fertility the family function “quasibinomial” was used due to overdispersion of the data, together with the link function “logit”. For the number of ovules the family function “quasipoisson” was used due to overdispersion of the data together with the link function “log” (natural logarithm). For single seed mass the family function “gamma” was used together with the link function “inverse”.

All analyses were carried out in R (R Developmental Core Team 2010). Graphs were produced in R using the ggplot2-package (Wickham 2009) and the grid-package (Murrell 2005).

Results

Results of the Pilot Study

In the pilot study, the hypothesis of transgenerational stability of the level of apomixis was tested. Therefore, sexual *H. pilosella* and apomictic *H. aurantiacum* were crossed. Hybrid offspring was tested for apomixis by decapitation, which removes anthers and stigma, thereby preventing pollination (Koltunow et al. 1995). To ensure apomictic origin, apomictic plants were propagated from seeds of decapitated capitula only. Two generations were grown and phenotyped. Unexpectedly, apomictic fertility changed over apomictic generations ($F_{2,6}$ = 16.7, p = 0.011, Figure 2A). Apomictic generation 1 (A1) and apomictic generation 2 (A2) increased their apomictic fertility compared to the original hybrid (t = 4.2, p = 0.006 and t = 3.0, p = 0.026, respectively). Furthermore, A2 plants decreased their apomictic fertility compared to A1 (t = -2.7, p = 0.036), indicating that this phenotype is not stable over apomictic generations.

Changes in fertility were not due to a different number of ovules ($F_{2,6}$ = 4.2, p = 0.072, Figure 2B). Neither has the single seed mass changed with fertility ($F_{2,6}$ = 0.24, p = 0.794, Figure 2C).

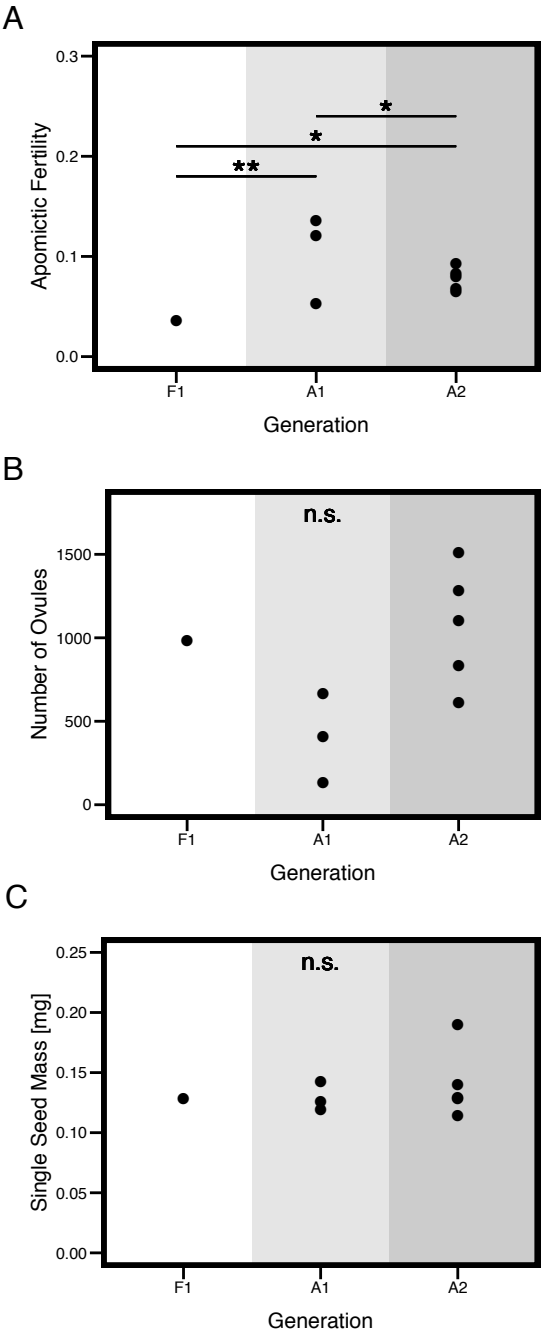


Figure 2. Phenotypic Changes Over Apomictic Generations

A) Change of apomictic fertility of one apomictic hybrid across two apomictic generations. Generations were grown subsequently. Number of dots corresponds to number of plants measured. Apomictic fertility increases from generation F1 to generation A1 ($t = 4.2$, $p = 0.006$) followed by decrease to A2 ($t = -2.7$, $p = 0.036$). Both generations have a higher apomictic fertility than the original hybrid (A2 compared to F1: $t = 3.0$. $p = 0.026$). Apomictic fertility – number of seeds/number of ovules from an unfertilized or decapitated capitulum. **B)** The number of ovules does not change across apomictic generations ($F_{2,6} = 4.2$, $p = 0.072$). **C)** Single seed mass does not change across apomictic generations ($F_{2,6} = 0.24$, $p = 0.794$).

Grey backgrounds – different apomictic generations; n.s. – not significant; · – $p < 0.1$; * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$

Unfortunately, only one apomictic interspecific hybrid was recovered from the original interspecific cross. Moreover, A1 and A2 were grown subsequently. This could result in biases. Nonetheless, an experiment using intraspecific hybrids to avoid possible confounding effects due to interspecific hybridization was planned (see Materials and Methods). By crossing we generated new apomictic lines with different levels of apomictic fertility. These apomictic lines were propagated apomictically over two generations, always growing the different generations at the same time.

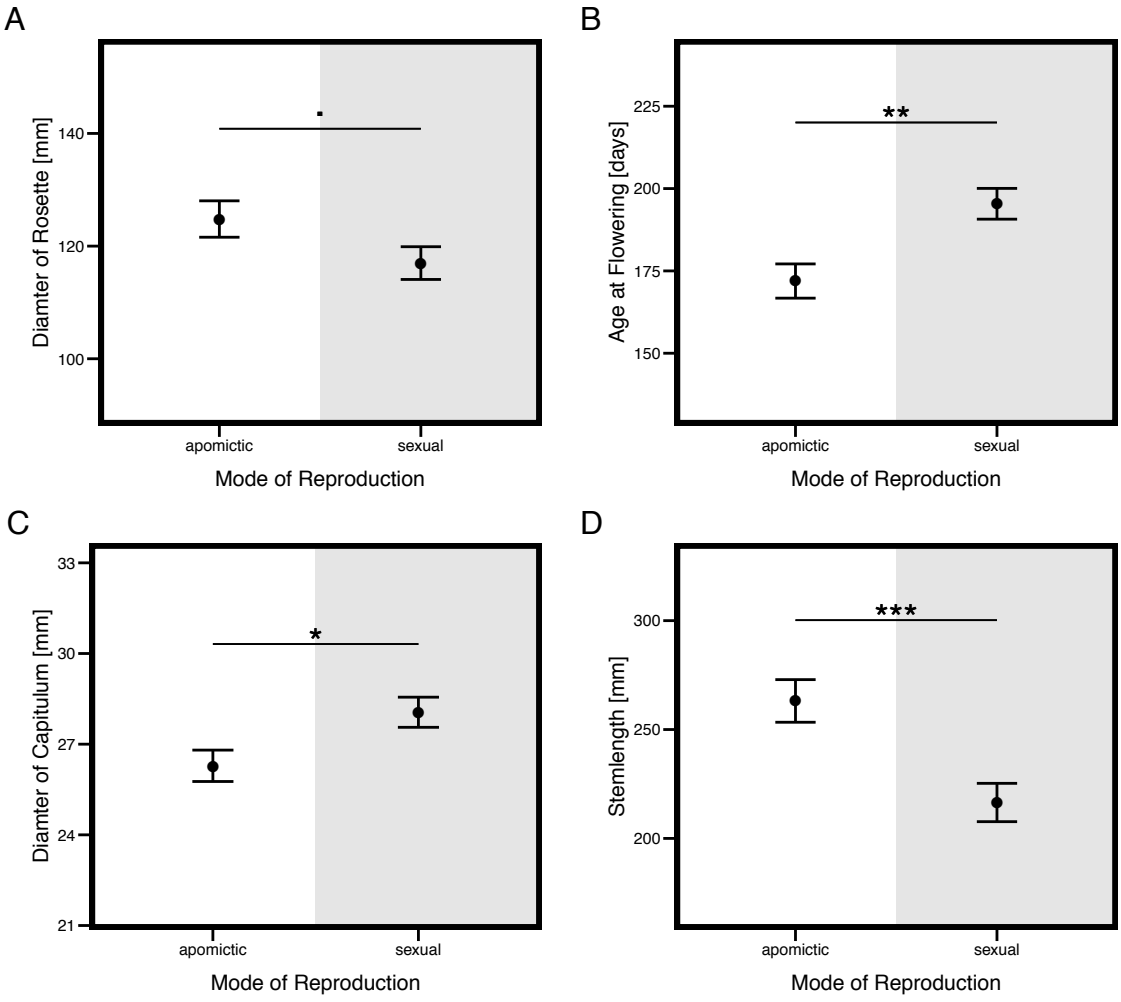
Results of Apomictic and Sexual Siblings (F1)

We have generated 113 new lines, 51 are apomictic while 62 are sexual. Due to the crossing scheme, apomicts and sexuals are full siblings.

The diameter of the rosette at flowering, which is a measure of growth, is slightly larger for apomicts than for sexuals ($F_{1,112}$ = 3.2, p = 0.075; Figure 3A). Furthermore, apomicts flower earlier than sexuals ($F_{1,112}$ = 11.3, p = 0.001; Figure 3B), which indicates that apomicts grow faster.

The diameter of the capitulum at the day of opening is smaller for apomicts than for sexuals ($F_{1,112}$ = 6.0, p = 0.015; Figure 3C). This phenomenon is reminiscent of the selfing syndrome. The selfing syndrome describes the morphological difference of flowers in selfing and outcrossing species (Sicard and Lenhard 2011).

Apomicts appear to have longer stems ($F_{1,112}$ = 12.6, p < 0.001; Figure 3D), which is a proxy for seed dispersal, suggesting that apomicts are able to further disperse their seeds than sexuals.



Apomictic Fertility in Generation A1

Apomictic fertility would be the important trait for agriculture. It can be translated to yield and is important for seed producers. Apomictic fertility varied from median values of 0.075 up to 0.617 in the generated apomictic lines in generation A1 (Figure 4). This variation shows the facultative nature of apomixis in a single species and indicates genetic determinants of apomictic fertility.

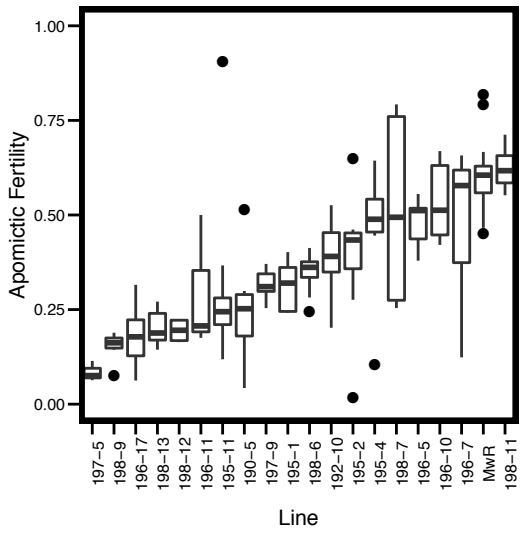


Figure 4. Variation of Apomictic Fertility in Different Genotypes
Median apomictic fertility varies from 0.075 to 0.617 in different lines of generation A1. The lines presented were also grown in generation A2. This variation reflects the facultative nature of apomixis and suggests genetic determinants of apomictic fertility. Boxes represent the inter-quartile range (IQR), the box-dividing bar represents the median. Whiskers extend to 1.5*IQR. Dots are outliers (> 1.5*IQR).
MwR is the line of the high apomixis father

Segregation of *LOP1*

In generation F1 we 51 apomicts and 62 sexuals segregated. Apomixis was determined by decapitation followed by seed set, and therefor by *LOP1*. A single locus is expected to segregate 1:1. *LOP1* segregated 1:1 in generation F1 ($F \sim binom(\pi = 0.5) = 0.17$). In generation A1 ploidy was determined by flow cytometry. Half of the plants were expected to be offspring of genotype *loa LOP* and therefore triploid. Only 3 of 71 plants were triploid ($F \sim binom(\pi = 0.5) < 0.001$). There is a bias against n + 0 offspring.

Figure 3. Phenotypic Differences Between Apomictic and Sexual Siblings

A) Apomicts have slightly larger rosettes ($F_{1,112} = 3.2$, $p = 0.075$). **B)** Apomicts flower earlier than sexuals ($F_{1,112} = 11.3$, $p = 0.001$). **C)** Apomicts have a smaller capitulum ($F_{1,112} = 6.0$, $p = 0.015$), which can be attributed to the selfing syndrome. **D)** Apomicts have longer stems ($F_{1,112} = 12.6$, $p < 0.001$).
Grey background – sexuals; n.s. – not significant; · – $p < 0.1$; * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$

State of the Experiment at the End of this Thesis

By the time this thesis is handed in, growing generations A1 and A2 together with the apomictic parents is finished, as is phenotyping traits at different developmental stages. Phenotyping traits concerning seeds (number of seeds, number of empty seed shells, number of ovules, apomictic fertility, mass of seeds, mass of empty seed shells, mean seed mass, Darwinian fitness) is pending. All statistical analyses are pending. Furthermore, genotyping to proof clonal identity of apomictic generations is pending.

Conclusions

F1 apomictic and sexual siblings differ in some phenotypes. Some of these lines were used in the Competition Siblings experiment, in which these differences were investigated in more detail (see chapter 2, Competition Siblings, of this thesis). The differences in the size of the floral display indicates that the selfing syndrome (reduced floral display in selfing species compared to outcrossing species) (Sicard and Lenhard 2011) also occurs in apomicts. For further discussion, please refer to chapter 2 of this thesis.

The apomictic lines generated vary in their apomictic fertility, which shows the facultative nature of apomixis in a single species. It further indicates that apomixis is a quantitative trait which is determined genetically. Of the 19 phenotypes which will be analyzed, apomictic fertility is the most important trait: First, seeds are the food produced by crop plants, second, apomictic fertility indicates the magnitude of reproductive assurance (Darwin 1862, Baker 1955, Stebbins 1957 this thesis, Darlington 1958, Tomlinson 1966) and third, it determines how much clonal progeny can be produced. Therefore, apomictic fertility is of importance for both farmers and seed producers.

The prospects of apomictic crop plants are very promising. However, it is still unknown if phenotypes can be fixed via apomixis. The experiment presented here will provide answers to this question. Furthermore, genetic and epigenetic contributions to varying phenotypes can be determined. The results of this experiment will influence apomictic breeding programs and contribute to our understanding of asexual reproduction in evolution.

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General Discussion

In this PhD thesis I aimed to characterize the relationship between the success of invasive plants and their mode of reproduction. This main objective was subdivided into analyzing: i) the role of apomixis in invasion, ii) the advantages of apomixis in succession, and iii) the fixation of phenotypes by apomixis over several generations. To address these objectives, a field study (Chapter 3), common garden (Chapters 1, 2), and greenhouse experiments (Chapter 4) were carried out.

Hieracium pilosella L. is native to Europe and it became invasive in New Zealand, where it is considered a pest (Day & Buckley, 2010; Murphy, 1878; Scott *et al*, 1990; Connor, 1992). Invasion has also recently been reported for Patagonia (Krahulcová & Krahulec, 2011). In both cases the cytotype is an apomictic pentaploid (aP5, Krahulcová & Krahulec, 2011; Houliston & Chapman, 2004; Chapman *et al*, 2000). To understand the role of apomixis in the invasion process, we compared apomictic and sexual lines under different competition settings in three common garden experiments, using different plant materials. Although the invasive apomictic lines from New Zealand out-performed the sexual European lines, sexuals were less influenced by different competition settings than apomicts. These results suggest that the European sexual lines were selected for stable reproduction, while the invasive New Zealand lines were selected for vigorous growth and spread (Chapter 1).

Further analysis using apomictic and sexual siblings revealed that the invasive success of apomictic lines in New Zealand could not be exclusively attributed to apomixis. In fact, we could not detect a fitness advantage of apomicts *per se*. Moreover, the whole genotype was affecting the phenotypes and the competitiveness of both apomicts and sexuals (Chapter 2). These results indicate that selection, besides acting on some genes underlying certain traits, also acts on the genome as a whole. Nevertheless, selection will ultimately result in genotypes that have a beneficial combination of alleles. Disruption of beneficial allele combinations by hybridization can result in a fitness loss (destabilizing hybridization, Lynch, 1984), a finding that is supported by our results (Chapters 1 & 2). We interpret this fitness loss as the hidden cost of sex. Furthermore, genotypic diversity contributes to stability in competition. In between-species competition, a stronger decrease in the number of stolons was observed for populations with a lower number of genotypes (2 versus 4 genotypes, Chapter 2). Genotypically diverse populations are therefore more resistant to invasion (Vellend, 2006; Lipowsky *et al*, 2011).

Genotypic diversity can be achieved either by different apomictic clones in a pure apomictic population or by apomictic and sexual genotypes in a mixed population, the latter being the common case in natural habitats (van Dijk, 2003; Mráz *et al*, 2008; Chapter

3). This might be explained by the facultative nature of apomixis (Asker & Jerling, 1992; Koltunow & Grossniklaus, 2003). In fact, we have shown that apomixis in *H. pilosella* is facultative and that the level of apomixis varies in a natural population on a glacial foreland (Chapter 3). We attribute this variation to the occurrence of different apomictic genotypes, since new apomictic lines, which we generated by crossing, also showed variation in apomictic fertility (Chapter 4). The new apomictic lines are the offspring of two apomictic fathers, which had different apomictic fertility. The two responsible loci for apomixis in *H. pilosella*, *LOA* and *LOP* (Catanach *et al*, 2006; Koltunow *et al*, 2011), are carried by all new apomictic lines. The exhibited variation in apomictic fertility indicates that apomixis is a quantitative trait, and, hence, should be controlled by more than the two loci of *LOA* and *LOP*.

As a quantitative facultative trait, apomixis provides reproductive assurance (Stebbins, 1957; Baker, 1967; 1965; Tomlinson, 1966; Darlington, 1958; Chapter 2) on the one hand, and, being facultative, generation of new genotypes by sexuality on the other. Indeed, we found more apomictic offspring at early stages of succession (Chapter 3), and apomicts exhibited a more stable fertility across different competition settings (Chapters 2). This supports Tomlinson's (1966) prediction about the reproductive advantage of parthenogens and reproductive assurance in sparse population densities. Furthermore, facultative apomixis is a trait which fulfills the requirements of a successfully adapting species as suggested by Stebbins (1957). Not only did we find support for Stebbins' prediction, we furthermore found evidence for Baker's law (Baker, 1955), which states that a single apomictic or self-fertilizing individual is sufficient to found a new population. The majority of plants in the natural population was hexaploid. Therefore, triploid plants must be n + 0 offspring (polyhaploid, half the ploidy of the mother plant). Even though we could not recover n + 0 seeds in the natural population, we found two patches of triploid plants, which must be the result of successful colonization by n + 0 offspring (Chapter 3). As a matter of fact, n + 0 offspring are biased against, since they do not segregate 1:1 as would be expected from an independent single locus (Chapter 4). They occur from a cross over between the sexual (meiosis) and the apomictic (parthenogenesis) pathway and are thus rare. However, a single, triploid apomictic individual is sufficient to found a new triploid population. The fact that two triploid patches occur in the natural population of a glacier foreland supports Baker's law.

Apomixis being a quantitative trait within a species was interpreted by Darlington (1958) as a characteristic of a still evolving species, and that the facultative nature of apomixis is due to recombination. As an example he used meiotic diplospory. Furthermore,

he assumed that evolution of apomixis in a species leads to obligate apomixis, which does not have residual sexuality. Such a species would be unable to adapt to new environments by generating new genotypes and, in addition, would accumulate mutations without the possibility of removing them (Muller, 1932). This led Darlington to conclude that apomixis is a blind alley of evolution (Darlington, 1958). However, changing environments and an increased number of mutations might act against the evolution of obligate apomixis, which would explain the occurrence of mixed natural populations of apomicts and sexuals. Furthermore, the facultative nature of apomixis ensures the production of sexual offspring and, unless there is a segregation distortion against sexuality, purely sexual offspring will always be produced together with facultative apomictic offspring due to segregation.

Besides segregation, sexuality means recombination, which in turn allows the removal of mutations from the genome. Kondrashov (1985) modeled allele frequencies of apomicts and sexuals in facultative apomicts as a function of genome degradation rate, based on the 2-fold cost of sex (Smith, 1978; Maynard-Smith, 1971). Genome degradation rate *v* is the number of deleterious mutations per generation divided by the square root of the critical number of mutations, with “critical” referring to the extinction of a genotype carrying this critical number of mutations. Above $v = 1.25$ obligate sexuality is established, and below $v = 0.125$ obligate apomixis is established. Between these values apomixis alleles are in equilibrium with sexuality alleles. In competition between apomixis and sexuality, the advantage of apomixis (*c*) grows linearly with the level of apomixis (*z*) in a population. Selection against apomixis depends on *v* (f_i). The equilibrium between apomixis and sexuality is likely to be reached at the maximum value of $c\text{-}f_i$. This value determines the value of *z* of a population (Figure 1).

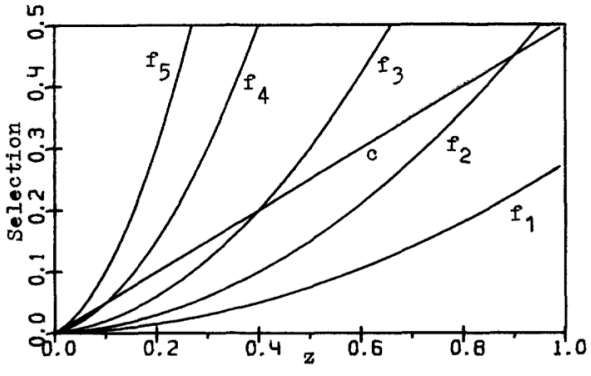

Figure 1. A scheme of factors acting for and against apomixis
Level of apomixis (z), advantage of apomixis (c), and selection against apomixis (fi) at certain genome degradation rate. The growth of i corresponds to the growth of the genome degradation rate.
Figure take from (Kondrashov, 1985).

Kondrashov’s (1985) model predicts that obligate apomixis can only be established under slow accumulation of mutations. We can assume that mutation rates are not constant over time in a natural population, and it is likely that mutation rates are in a range which results in a genome degradation rate under which apomixis and sexuality are in

equilibrium. This consideration is supported by the occurrence of established apomictic genotypes together with new apomictic and sexuals genotypes in a natural population of a facultative apomictic species. In other words, natural populations have a genetic diversity consisting of genotypes that already have been selected, and new genotypes which have not yet been selected. The frequencies of established apomictic and of new apomictic and sexual genotypes are determined by the level of apomixis, which in turn is highly variable. The fact that we find different levels of apomixis in the natural population indicates that apomicts and sexuals are, indeed, in a dynamic equilibrium.

Apomixis would only be a blind alley of evolution if it were obligate, and therefore had no possibility of removing mutations. Our data from a natural population interpreted with Kondrashov's (1985) model predicts selection against obligate apomixis and, therefore, against a blind alley. The fact that we found mainly low levels of apomixis in the natural population further supports this argument (Chapter 3).

Reproductive assurance is a selective force which acts to favor apomixis, while genome degradation rate is a selective force acting against apomixis. Our results point toward a dynamic equilibrium of apomixis and sexuality in natural populations. Which one is prevalent depends on the environment and on the genotypes present in the population, with selective forces shifting this equilibrium. In contrast to a purely sexual population, which generates a new set of genotypes in each generation, a facultative apomictic population reproduces a new set of genotypes plus already successful (since they reproduced) and established genotypes. Due to reproductive assurance, the established genotypes are available for creation of new genotypes in each generation, and therefore provide a basic "stock" of successful alleles. Selective forces select new successful genotypes, which, if they are apomictic, are again available in the next generation as a genetic resource, which is not the case in purely sexual populations.

This provision of successful allele combinations each generation via apomicts is the main advantage of apomixis, not only in colonization or succession, but also in established populations. We can think of apomixis as a mechanism to preserve and provide combinations of beneficial alleles, thereby assisting in the creation of new successful genotypes.

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Appendices

Contributions to other publications

During my PhD thesis I have contributed to the following publications:

- H Lindner, M T Raissig, **C Sailer**, H Shimosato-Asano, R Bruggmann, and Ueli Grossniklaus, SNP-Ratio Mapping (SRM): Identifying Lethal Alleles and Mutations in Complex Genetic Backgrounds by Next-Generation Sequencing, Genetics, 2012

- Tufail Bashir, **Christian Sailer**, Nitin Loganathan, Hemadev Bhoopalan, Christof Eichenberger, Ueli Grossniklaus, Ramamurthy Baskar, Hybridization alters spontaneous mutation rates in a parent-of-origin-dependent fashion in *Arabidopsis thaliana*, Plant Physiology, submitted, 2013

- Amit Kumar Singh, Tufail Bashir, **Christian Sailer**, R. Anantha Maharasi, Shanmuhapreya Dhanapal, Ueli Grossniklaus and Ramamurthy Baskar, Parental Age Effects on Somatic Mutation Rates in Flowering Plants, Ageing Cell, submitted, 2013

For Lindner et al. I have calculated the minimum coverage required to differentiate between 1:1 and 1:3 segregation. Furthermore, I have written the R-script to identify candidate causative SNPs.

For Bashir et al. I have analyzed all data, constructed all figures and all tables. Furthermore, I have written the statistical analysis section and parts of the results section of the publication.

For Singh et al. I have analyzed all data and constructed all figures. Furthermore, I have written the statistical analysis section of the publication.

The abstracts of these publications are printed on the following pages.

SNP-Ratio Mapping (SRM): Identifying Lethal Alleles and Mutations in Complex Genetic Backgrounds by Next-Generation Sequencing

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Abstract

We present a generally applicable method allowing rapid identification of causal alleles in mutagenized genomes by next-generation sequencing. Currently used approaches rely on recovering homozygotes or extensive backcrossing. In contrast, SRM allows rapid cloning of lethal and/or poorly transmitted mutations and second-site modifiers, which are often in complex genetic/transgenic backgrounds.

Hybridization alters spontaneous mutation rates in a parent-of-origin-dependent fashion in *Arabidopsis thaliana*

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Abstract

Over 70 years ago, A.H. Sturtevant observed increased spontaneous mutation rates in Drosophila hybrids. To this date, the genetic basis of this phenomenon is not well understood. The model plant *Arabidopsis thaliana* offers unique opportunities to study the types of mutations induced upon hybridization and the frequency of their occurrence. Understanding the mutational effects of hybridization is important, as many crop plants are grown as hybrids. Besides, hybridization is important for speciation and effects on genome integrity could be critical, as chromosomal rearrangements can lead to reproductive isolation. We examined the rates of hybridization-induced point mutations, frameshift mutations, and homologous recombination in intraspecific *Arabidopsis* hybrids using a set of transgenic mutation detector lines carrying mutated or truncated versions of a reporter gene. We found that hybridization alters the frequency of different kinds of mutations. In general, Col x Cvi and Col x C24 hybrid progeny had a decreased rate of T→G transversions but an increased rate of C→T transitions. In Col x C24 hybrids there is a trend for increased homologous recombination rates, while in Col x Cvi hybrids this rate is decreased. Surprisingly, no significant change in frameshift mutation rates was observed in hybrids. The genetic distance of the parents had no influence on mutation rates in the progeny, as closely related accessions on occasion displayed higher mutation rates than accessions that are separated farther apart. However, reciprocal hybrids had significantly

different mutation rates, suggesting parent-of-origin-dependent effects on the mutation frequency.

Parental Age Effects on Somatic Mutation Rates in Flowering Plants

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Summary

In humans, it is well known that the parental reproductive age has a strong influence on mutations transmitted to their progeny. Meiotic non-disjunction is known to increase in older mothers and point mutations tend to go up with paternal reproductive age. Hence, it is clear that the germinal mutation rates are a function of both maternal and paternal age in humans. In contrast, it is unknown whether the parental reproductive age has an effect on somatic mutation rates in the progeny, as these are rare and difficult to detect. To address this question, we took advantage of the plant model system *Arabidopsis thaliana*, where mutation detector lines allow for an easy quantitation of somatic mutations, to test the effect of parental age on somatic mutation rates in the progeny. We found that the T-C transition point mutations increase in the progeny with the increased parental reproductive age. Similarly, frameshift mutations and transposition events increase in progeny of older flowers, an effect that is stronger through the maternal than the paternal side. However, homologous recombination events in the progeny decreased with the age of the parents in a parent-of-origin-dependent manner. Our results clearly show that parental reproductive age affects somatic mutation rates in the progeny and, thus, that some form of age-dependent information is transmitted through the gametes.

Genotyping Approaches

Two genotyping approaches have been applied and tested. AFLPs (Amplified Fragment Length Polymorphisms, Vos et al. 1995), and Simple Sequence Repeats (SSRs). SSR markers for *H. pilosella* are published (Zini and Komjanc 2008). Both methods turned out to give un-reproducible results. We assume that this is due to some compounds which were neither removed by the CTAB-DNA-extraction method (Stewart and VIA 1993), nor by Qiagen DNeasy technology. Indeed, *Hieracium pilosella* L. is medical herb and it contains antioxidative compounds, such as chlorogenic acid, umbelliferones and apigenin-7-O-glucoside (Stanojevic et al. 2009). We assume that the highest DNA quality ($CsCl_2$ gradient centrifugation) is necessary for reproducible AFLPs and SSRs results. Poorer DNA qualities result in stochasticity, which in case of AFLPs is amplified two times. I have extensively tested and tried to optimized these two methods with CTAB and Qiagen DNA quality without success.

So far only a technique based on next generation sequencing (NGS), 2b-RAD (Wang et al. 2011), was successfully applied by Eli Meyer, at that time a post doc in Thomas Juenger's group. *H. pilosella* has an estimated genome size of 1C = 3 Gbp (Suda et al. 2007). An NGS method which reduces the complexity of the genome is therefore essential for successful genotyping by sequencing. RAD (Restriction site Associated DNA) reduces the genome complexity by using specific restriction enzymes. In the case of 2b-RAD a type IIB restriction enzyme, which cuts upstream and downstream of its specific recognition site, is used. The big advantage of this method is the specific size of the fragments, which are sequenced. Combining this technique with RTR (Reduced Tag Representation) allows for further reduction of the genome complexity from 1/16 to 1/4096. This reduces the amount of sequenced bases necessary to reach the minimum of 20-fold coverage (Wang et al. 2011), for Illumina sequencing. Sequencing on the Illumina platform has shown to be low in errors, has tools available for analysis and was applied successfully on large genomes (e.g. Elshire et al. 2011).

Since we found that apomicts are rare and the variation of the level of apomixis being big in the natural population of the glacial foreland of Morteratsch, we decided to not genotype individual plants. We assumed that the additional information from genotypes will not be worth the effort and costs.

GPS coordinates (swiss grid) of sampled patches at the glacial foreland of Morteratsch

Population indicates the given patch name, YAD are the Years After Deglaciation. “0” indicates missing GPS data.

Population	Longitude	Latitude	YAD	Deglaciated since
1068	0	0	51	1960
1076	791975	145658	51	1970
1078	791965	145716	51	1960
327	791863	145441	51	1970
363	791897	145516	51	1970
375	791925	145546	51	1970
379	791844	145572	51	1970
394	791850	145611	51	1970
403	791869	145632	51	1970
414	791856	145676	51	1960
460	791824	145800	51	1960
KE1	791861	145452	51	1970
KS2	791850	145592	51	1970
1045	792163	146236	71	1940
1050	792189	146174	71	1940
1052	0	0	71	1950
672	791758	145985	71	1940
676	791751	145997	71	1940
1027	792247	146563	91	1920
1030	792249	146396	91	1920
1031	792249	146545	91	1920
1033	792246	146539	91	1920
1035	792262	146532	91	1920
750	791779	146171	91	1920
768	791806	146242	91	1920
782	791815	146272	91	1920
793	791837	146320	91	1920
800	791878	146340	91	1920
802	791881	146341	91	1920
310	792172	146824	111	1900
311	792171	146826	111	1900
317	792132	146722	111	1900
A1	792316	146739	111	1900
T4	792017	146569	111	1900
TE1	792015	146575	111	1900
TS3	791999	146564	111	1900
121	792201	147029	131	1880
127	792211	147025	131	1880
155	792251	147025	131	1880
163	792251	147062	131	1880
164	792273	147054	131	1880
177	792229	147000	131	1880
185	792196	146983	131	1880
200	792223	146963	131	1880

Population	Longitude	Latitude	YAD	Deglaciated since
208	792188	146975	131	1880
239	792231	146915	131	1880
243	792216	146905	131	1880
251	792217	146893	131	1880
8	792279	147218	154	1857
16	792250	147160	154	1857
23	792253	147208	154	1857
24	792241	147202	154	1857
25	0	0	154	1857
1007	792396	147146	154	1857
1011	792407	147120	154	1857
1012	792409	147124	154	1857
1013	792411	147127	154	1857
1020	792425	147018	154	1857

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